THE AMOUNT OF PLASMA TRAPPED BY RED CELLS IN CENTRIFUGED AND SEDIMENTED BLOOD

by PEGGY CLARK[1] AND R. J. WALSH

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

(*Accepted for publication 17th August, 1960.*)

SUMMARY.

An accurate method for dividing red blood cell columns has been devised. Blood is placed in P.V.C. tubing which is sealed at one end with a glass bead. This tubing is inserted into a piece of glass tube before centrifugation, and later the contents can be frozen before the tubing is divided. The method was used to measure the amount of plasma trapped with the red blood cells. From these measurements the ratio of the true to the observed haematocrit value was found to be 0·94 at centrifugal forces greater than 1,430 *g*. The ratio decreased with decreasing relative centrifugal force, but variations in the haematocrit value did not alter the correction factor. The amount of plasma trapped in centrifuged and sedimented normal human blood decreased from top to bottom of the red cell columns, but the amount in different sections of the columns became more uniform as the time of centrifugation or of sedimentation increased. A blood sample with a high sedimentation rate showed a different pattern on sedimentation.

INTRODUCTION.

Some plasma is always found amongst the red cells in the centrifuged column of blood when the haematocrit value is determined. Several techniques have been used to determine the amount of trapped plasma and then to calculate the ratio of the correct to the observed haematocrit values. The ratio is essential when the total blood volume is calculated from the haematocrit value together with the measured red cell volume or plasma volume. A simple technique, to be described in this paper, has enabled further investigations to be made of the amount of trapped plasma and of some of the factors which influence it.

The technique was developed in the first instance to provide a method for separating and studying sections of the red cell columns. Removal of successive layers by pipetting is difficult and unsatisfactory (Clark, 1959) and other methods have been sought. Randolph and Ryan (1950) devised an apparatus for cutting "Lusteroid" centrifuge tubes without distorting the tube and without spilling

[1] This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

the contents. Borun, Figueroa and Perry (1957) also used plastic tubes but froze the contents before cutting. The new technique is based on these earlier methods, but is easier to perform.

TECHNIQUE.

The apparatus consists of:—

(a) Glass tubing, 12 cm. long, with an inside diameter of 4·8 mm. The ends of this tube are smoothed in a flame and the interior surface of the tube is covered with *paraffinum molle*.

(b) Plastic tubing (P.V.C.) approximately 13 cm. long with an inside diameter of 3·2 mm. and wall thickness of 0·8 mm.

(c) Spherical glass beads 4 mm. in diameter.

A glass bead is inserted into one end of the P.V.C. tubing, which is then inserted into the glass tubing until the bead-expanded end makes firm contact with the end of the glass tube. Blood or other fluid is placed in the plastic tube with a long pipette, as in filling a Wintrobe haematocrit tube. If desired, another bead can be used to seal the upper end of the plastic tube. Centrifugation results in the glass tube being pressed against the lower glass bead to improve the seal. The apparatus is illustrated in Fig. 1.

In the experiments described below human blood samples were mixed with radio-iodinated human serum albumin (obtained from the Radiochemical Centre, Amersham, England) and plasma trapped in the red cells was subsequently measured using an "EKCO" scintillation counter with "end-on" crystal arrangement.

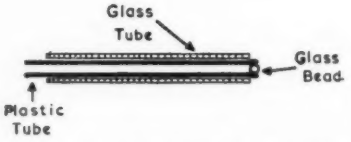

Fig. 1. The complete apparatus described in the text.

Radioassays were made of the total red cell column and of portions of the column in different experiments. When the total column was assayed the plastic tube was removed from the glass tube and the upper end sealed with a bead. The tube was placed in the horizontal position, and without delay divided with a razor blade at the top of the red cell column. It was possible to make this division without distorting the tube.

When portions of the red cell column were assayed the whole assembly of glass and plastic tube with centrifuged blood was placed in a bath of methylated spirit and solid carbon dioxide until frozen. During freezing considerable expansion of the red cells occurred and the cell-plasma interface was distorted so that a clean-cut division between cells and plasma would not have been possible and it was necessary to discard the section of red cells adjacent to the plasma. The plastic tube was removed from the glass tube and, while the contents were still frozen, sections of the red cell column each 12 mm. long from the bottom of the tube upwards were cut with a razor blade.

The centrifuge employed was one commonly available in clinical laboratories and attained a maximum speed of 3,650 r.p.m. with six buckets loaded. It had a radius from the centre to the top of the bead when the tube was in position of 13·8 cm. and the speeds were adjusted with the aid of a tachometer. The relative centrifugal force (R.C.F.) was calculated from the formula:

$$\text{R.C.F.} = 11{\cdot}18 \times 10^{-6} \times r \times \text{r.p.m.}^2$$

where r is the radius in centimetres.

The percentage of plasma trapped in the red cell column was calculated as follows:

$$\text{Percentage plasma trapped} = \frac{\text{c.p.m. in red cells}}{\text{Total c.p.m. in plasma and red cells}} \times 100.$$

The percentage increased at speeds below 3,000 r.p.m. and varied between 4·6 and 6·9 p.c.

The true haematocrit was calculated from the formula:

$$\text{Haematocrit} = 100 - \frac{(100 - \text{Hct})(100 + \% \text{ plasma trapped})}{100}$$

where Hct is the observed haematocrit percentage. From this value the ratio of the true haematocrit to the observed haematocrit was calculated for each set of results. This ratio was identical for 3,000 and 3,500 r.p.m., but less at the lower speeds.

EXPERIMENTAL PROCEDURES AND RESULTS.

Trapping of plasma after centrifugation at different speeds. Radio-iodinated human serum albumin (0·2 ml. RIHSA containing approximately 5 μc) was added to 25 ml. of heparinised blood. After thorough mixing, 24 plastic tubes as described were filled and centrifuged at different speeds. After division the tubing containing the plasma and red cell layers was placed in separate plastic containers for radioassay and the tubing washed through with 10 ml. of distilled water. The tubing was then cut into small pieces, which were left in the containers.

TABLE 1.

Trapping of plasma at different speeds of centrifugation.

R.P.M.	R.C.F.	Number of tubes	Haematocrit p.c. (Mean ± S.D.)	C.P.M. in plasma (Mean ± S.D.)	C.P.M. in red cells (Mean ± S.D.)	Plasma in red cells p.c.	True Hct / Obs. Hct
2,000	635 *g*	5	44·6 ± 0·22	43,110 ± 739	3,192 ± 326	6·89	0·91
2,500	990 *g*	6	44·0 ± 0·56	44,557 ± 1416	2,654 ± 277	5·63	0·92
3,000	1,430 *g*	5	43·9 ± 0·50	44,408 ± 806	2,105 ± 463	4·42	0·94
3,500	1,945 *g*	6	43·3 ± 0·63	43,785 ± 526	2,136 ± 436	4·64	0·94

The results of measuring the amount of trapped plasma in the red cell layer are shown in Table 1. There was a decrease in the mean haematocrit value from 44·6 to 43·3 as the R.C.F. increased, but Koeppe's criterion of complete cell packing as described by Wintrobe (1956) was not observed in any of the tubes.

Plasma trapping with varying haematocrit values. Blood with radio-iodinated human serum albumin was placed into three glass centrifuge tubes so that the tubes contained 11 ml, 8 ml. and 5 ml. respectively. The tube with 11 ml. was centrifuged and 3·5 ml. of supernatant plasma removed and added to the tube containing 5 ml. of blood. All three tubes were well mixed by rotation for 5 minutes on a machine described by Dacie (1956) and six special haematocrit tubes were filled from each sample. The tubes were centrifuged at 3,000 r.p.m. for 30 minutes and the cell and plasma layers assayed for radioactivity.

The effect of varying haematocrit values produced as above on the amount of trapped plasma was investigated with a constant speed of centrifugation of

3,000 r.p.m. equivalent to a relative centrifugal force of 1,389 *g*. The results in Table 2 show that the amount of trapped plasma increased with increasing haematocrit values, but that the ratio of the true haematocrit to the observed haematocrit did not vary significantly.

TABLE 2.

Percentage of plasma trapped with varying haematocrit values. (3000 *r.p.m.*)

Number of tubes	Haematocrit (Mean±S.D.)	C.P.M. in plasma	C.P.M. in red cells	Plasma in red cells p.c.	True Hct / Obs. Hct
6	59·11±0·24	26,526±585	2,732±183	9·33	0·93
6	41·00±0·55	39,360±1,361	1,676±121	4·13	0·94
6	28·96±0·20	46,865±964	1,095±75	2·29	0·94

Plasma trapping at different levels of red cell column. Haematocrit tubes containing blood with radio-iodinated human serum albumin were centrifuged at different speeds for 30 minutes, then frozen. Three columns each approximately 12 mm. long were cut from each tube and placed in separate plastic containers for radioassay, 10 ml. of water being added to each container. After assay 2 ml. from each container were added to 10 ml. of water and the relative haemoglobin content of the solution was determined.

The results are shown in Table 3. The radioactivity (expressed as counts per minute) of the sections is plotted in Fig. 2 (a) against the relative centrifugal forces and lines are drawn through the mean values for the three sections, upper, middle and lower, of each tube.

TABLE 3.

Trapping of plasma at different levels of the red cell column and at different speeds of centrifugation

R.P.M.	Number of tubes	R.C.F. at bottom of section	Sections of red cell column	Haemoglobin in column compared with section 3	C.P.M. in section Mean±S.D.	C.P.M. (p.c.) in section compared with section 3
2000	6	510 *g*	upper	0·95	1186±35	162
		563 *g*	middle	0·99	958±38	131
		617 *g*	lower	1·00	734±40	100
2500	5	797 *g*	upper	1·03	859±54	158
		880 *g*	middle	0·99	735±54	135
		964 *g*	lower	1·00	545±27	100
3000	6	1147 *g*	upper	0·99	773±39	155
		1268 *g*	middle	1·01	672±63	134
		1389 *g*	lower	1·00	500±64	100
3650	4	1698 *g*	upper	0·96	653±43	159
		1877 *g*	middle	0·98	559±4	136
		2055 *g*	lower	1·00	411±8	100

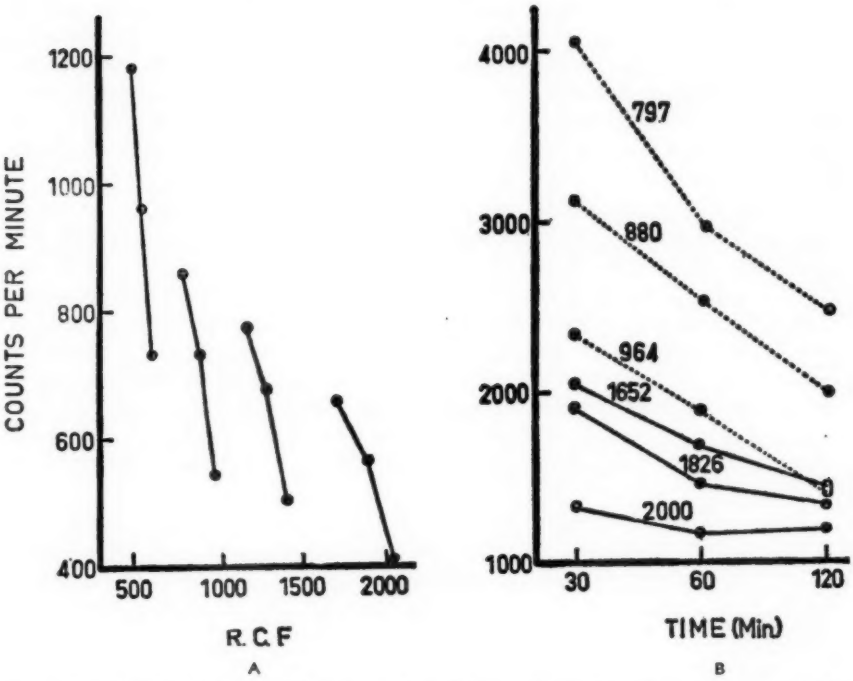

Fig. 2. Plasma trapping at different speeds and times of centrifugation. Each point is the mean of six results.

A The lines connect the points for the different levels in tubes, so that each line concerns the tubes spun at a fixed speed.

B The results shown by broken connecting lines were obtained when the tubes were centrifuged at 2,500 r.p.m., and those shown by continuous connecting lines at 3,600 r.p.m. The numbers opposite each line represent the R.C.F. values. The lines connect the same levels in different tubes.

The following points should be noted:—

(a) The degree of packing, as determined by the amount of trapped plasma, varies in different parts of the column. Packing is greatest at the bottom of the tube.

(b) An increase in the rate of centrifugation decreases the amount of trapped plasma in all parts of the column and the reduction occurs to approximately the same degree in all parts of the column. This is evident from the last column of Table 3.

(c) The variations in the haemoglobin content of the 12 mm. sections are due to errors in cutting the tubes rather than to variations in the amount of trapped plasma. However, the differences are not significant.

(d) There is not a direct correlation between the R.C.F. readings and the amount of plasma trapped in the sections. It is evident (Fig. 2a) from the lines drawn through the three points in the columns that the differences in the plasma trapped at different levels are reduced as the R.C.F. values increase, but they are not eliminated within the range of R.C.F. values used. There must, therefore, be some other factor or factors determining the amount of plasma trapped.

Plasma trapping with different times of centrifugation. A sample of heparinised blood containing radio-iodinated human serum albumin was used to fill the special tubes and after centrifugation for various times the tubes were frozen. Three 12 mm. lengths of the red cell column were cut from each tube and analysed for radioactivity as before. The results are shown in Fig. 2b, each point representing the mean of six determinations. In this instance, radioactivity expressed as c.p.m. was plotted against time of centrifugation. The experiment was performed at two speeds, 3,600 r.p.m. and 2,500 r.p.m. At 3,600 r.p.m. the haematocrit decreased from 46·8 p.c. after 30 minutes to 45·5 p.c. after 120 minutes; at 2,500 r.p.m. the decrease was from 49·5 p.c. at 30 minutes to 46·8 p.c. at 120 minutes.

The results in Fig. 2b show that at both speeds the amount of trapped plasma in different parts of the red cell column decreases as the time of centrifugation increases. It can also be shown (although not in the Figure) that the amount of plasma trapped in different levels of the column after 120-minute centrifugation at 3,500 r.p.m. is more nearly correlated with the R.C.F. values than were the amounts of trapped plasma after 30 and 60 minutes centrifugation. The factors preventing efficient packing of red cells are therefore minimised by prolonged centrifugation.

Plasma trapping in sedimenting red cells. Heparinised blood was obtained from an apparently normal blood donor (haematocrit 48 p.c.) and from a female patient with severe rheumatoid arthritis (haematocrit 39 p.c.). Radio-iodinated human serum albumin was added to each sample of blood and plastic tubes of the type used in the haematocrit experiments were filled to approximately 110 mm. with blood from each subject. The tubes were stood in the vertical position and the sedimentation rate measured at different time intervals. The contents of six tubes from each subject were frozen after one hour, six after two hours and six after four hours. Sections of the frozen columns, 12 mm. long, were cut from the bottom upwards and the radioactivity measured with the results shown in Table 4.

It can be seen that with the normal blood the amount of trapped plasma (a) becomes progressively less in each section with time, (b) increases from the bottom upwards at all three times, and (c) becomes more uniform in the sections at the later times, i.e., the difference between the bottom and the fifth sections is less at four hours than at one and two hours.

TABLE 4.

Plasma trapping in sedimenting red cells.

		Time of sedimentation		
		1 hour	2 hours	4 hours
Sedimentation rate (mm) Normal subject		15	35	40
Counts per minute in 12 mm sections of red cell column (From bottom upwards)	1	13,858	10,965	10,204
	2	15,058	11,654	10,177
	3	17,086	12,542	10,425
	4	18,578	14,183	10,875
	5	19,435	16,673	11,662
Sedimentation rate (mm) Rheumatoid arthritis subject		49	53	54
Counts per minute in 12 mm sections of red cell column (From bottom upwards)	1	8,022	7,127	6,667
	2	8,900	7,232	6,296
	3	8,559	6,704	5,834
	4	8,559	6,484	5,608

The blood from the patient with rheumatoid arthritis shows a different pattern with the following features:

(a) At all times the amount of trapped plasma in the sections varies much less than with the normal blood.

(b) At four hours there is a progressive reduction in the amount of plasma from the bottom upwards, i.e., the reverse of the findings with the normal blood sample. This trend is also evident at two hours.

(c) There is less reduction in the amount of plasma in corresponding layers at the various times, but this is probably because the sedimentation rate is higher.

It should be mentioned that less radio-iodinated albumin was added to the blood sample from the patient with rheumatoid arthritis. This is the reason why less radioactivity was found in the sections from the second sample.

DISCUSSION.

The method which has been described permits red cells to be separated accurately according to their position in a column of centrifuged blood. The technique is simple and it will allow further investigations of the relationship of the column position of the cells to their specific gravity and in turn to their age and physical and chemical properties (Clark, 1959). Modifications, such as larger bore tubes and more dilute blood samples, may be necessary for these applications.

The reliability of the technique was investigated by using it in a study of some physical aspects of the trapped plasma in a centrifuged column of blood. In general, the conclusions of other workers have been confirmed. Similar studies have been made with Evans Blue (Millar, 1925; Chaplin and Mollison, 1952), radioactive chromium (Ebaugh, Levine and Emerson, 1955) and radio-iodinated (I^{131}) albumin (Hodgetts, 1959; Owen and Power, 1953; Vasquez, Newerley, Yalow and Berson, 1952).

It has been shown by Millar (1925), Chaplin and Mollison (1952) and Hodgetts (1959) that the plasma in the total column, as measured by the haematocrit value, is reduced by increasing either the relative centrifugal force or the time of centrifugation. The results obtained in the present work show that the relative amounts of plasma trapped in different sections of the red cell column are also dependent on both the rate and time of centrifugation. It seems probable that red cells present a physical obstruction to the escape of plasma from the column and that the longer the time at a given centrifugal force, the smaller the amount of trapped plasma in any particular layer. The results also suggest that ultimately the amount of plasma in each layer throughout the red cell column would become directly related to the relative centrifugal force applied to the layer. In other words, instead of the pattern seen in Fig. 2a there would be a curve on which all points would fall.

From a practical point of view the findings suggest that for the particular centrifuge used and at speeds above 3,000 r.p.m. for 30 minutes, the true haematocrit can be obtained by using a correction factor of 0·94 (see Table 2). Chaplin and Mollison (1952) have, however, shown that the correction varies with the rate and time of centrifugation and have produced a curve for calculating the appropriate correction factor for use under different conditions. The correction factor is not related to the absolute haematocrit value, but changes with the speed of centrifugation.

The results with the sedimenting blood are interesting. With the normal blood the pattern is similar to that obtained when the tubes are centrifuged at low speeds for varying times. The sample of blood from the patient with rheumatoid arthritis had a rapid sedimentation rate and a different pattern was observed. The plasma was trapped more uniformly in all sections and ultimately the greatest amount was found in the lowest layer. Rouleaux formation is known to be intense in blood with a high sedimentation rate and the large aggregates of cells sediment rapidly. It appears that the large aggregates prevent the escape of plasma when the cells fall naturally.

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THE EFFECT OF CORN OIL AND OF COCONUT OIL ON THE ESTERIFICATION AND REMOVAL OF CHOLESTEROL BY RETICULO-ENDOTHELIAL CELLS

by A. J. DAY[1]

(From the Sir William Dunn School of Pathology, University of Oxford).

(*Accepted for publication 17th August, 1960.*)

SUMMARY.

Emulsions containing either corn oil or coconut oil were injected intraperitoneally into rats at the same time as cholesterol suspensions, in order to determine whether the presence of polyunsaturated or saturated fatty acids had any effect on the uptake, esterification and removal of cholesterol by reticulo-endothelial cells in the sternal lymph nodes.

Both corn oil and coconut oil increased the amount of cholesterol taken up by the sternal nodes. The amount of cholesterol esterified during the first 24 hours was less in the presence of coconut oil than in the presence of corn oil. The percentage ester, however, increased more in the coconut oil group after 24 hours, and by 10 days exceeded the percentage ester in the corn oil group.

The removal rate of total cholesterol from the nodes was not significantly affected by the presence of either coconut oil or corn oil.

INTRODUCTION.

The dietary administration of vegetable oils containing a large proportion of polyunsaturated fatty acids is known to reduce the serum cholesterol level (Kinsell, Partridge, Boling, Margen and Michaels, 1952; Ahrens, Tsaltas, Hirsch and Insull, 1955; Bronte-Stewart, Antonis, Eales and Brock, 1956). Since the feeding of such oils is associated with an increased formation of polyunsaturated fatty acid esters of cholesterol (Kinsell, Michaels, Friskey and Splitter, 1958; Mukherjee, Achaya, Deuel and Alfin-Slater, 1958) it is possible that more rapid metabolism and removal of these esters accounts, in part, for the reduction in serum cholesterol. Sinclair (1956) found that, in rats deficient in polyunsaturated fatty acids, cholesteryl esters containing saturated fatty acids were deposited in the skin; he postulated that these esters were not as readily removed as those formed when polyunsaturated fatty acids were present.

Cholesterol suspensions injected intraperitoneally are taken up readily by the reticulo-endothelial cells of rat sternal lymph nodes (French and Morris, 1960); previous work indicates that cholesterol is esterified in these cells and subsequently removed (Day and French, 1959; Day, 1960). The relationship

[1] Present address: Department of Human Physiology and Pharmacology, University of Adelaide, South Australia.

between esterification and removal, however, is not known. In the experiments now described, emulsions of corn oil or coconut oil were injected intraperitoneally into rats at the same time as cholesterol suspensions to determine whether these oils, with respectively high contents of polyunsaturated and saturated fatty acids, had an effect on the uptake, esterification and removal of cholesterol by reticulo-endothelial cells.

Materials and Methods.

Cholesterol suspensions. Finely divided suspensions were prepared by slowly adding a solution of cholesterol in acetone to boiling water. The acetone was removed and the solution concentrated by continued boiling. These suspensions were mixed with bovine albumin (Armour) and the concentration adjusted to contain 8 mg. cholesterol/ml. in 5 p.c. albumin solution. Prior to intraperitoneal injection this preparation was mixed with equal parts of either corn oil, coconut oil or control emulsion.

Corn oil and coconut oil emulsions were prepared by emulsifying 10 p.c. v/v of either corn oil or coconut oil in 5 p.c. dextrose solution with 0·5 p.c. Tween 60 added as emulsifying agent. A control "emulsion" containing Tween 60 in 5 p.c. dextrose solution but without oil was also prepared.

Experimental procedure. Male albino rats (200 gm.) were used. In the first experiment 72 rats were divided into 3 groups. The rats in the control group received intraperitoneally 2 ml. of a mixture containing equal parts of the cholesterol suspension in albumin solution and control "emulsion". The rats in the second and third groups received cholesterol suspension mixed with either corn oil emulsion or coconut oil emulsion respectively. At 24 hours, 4 days and 10 days after injection, 8 rats from each group were killed, the sternal nodes removed and the free and ester cholesterol content determined.

In the second experiment cholesterol suspensions mixed with either corn oil or coconut oil emulsions were given to two groups of 16 rats in each. Eight rats from each group were killed 24 hours after this injection, and the sternal nodes analysed as before. The remaining rats were given 3 further intraperitoneal injections of 1 ml. of corn oil or 1 ml. of coconut oil emulsion, without added cholesterol, 2, 4 and 7 days after the first injection. The rats were then killed at 10 days and the sternal nodes analysed.

In the third experiment cholesterol suspensions mixed with either corn oil or coconut oil emulsion were given to 32 rats. At 4 hr., 8 hr., 12 hr. and 24 hr. after injection 4 rats from each group were killed, and the free and ester cholesterol content of the sternal lymph nodes determined.

Extraction and determination of the free and ester cholesterol was carried out as previously described (Day and French, 1959).

RESULTS.

The amounts of free and ester cholesterol present in the nodes after the uptake of each of the three preparations in Exp. 1 is shown in Table 1. With both corn oil and coconut oil the uptake of cholesterol during the 24 hours following injection was greater than that in the control group. Ester cholesterol was present in the nodes within 24 hours in all the rats. The percentage ester was significantly less with coconut oil than with the corn oil ($p < 0{\cdot}05$).

Fig. 1A shows the changes in the percentage ester in the corn oil and coconut oil groups during the subsequent course of the experiment. By 4 days in both these groups the absolute amount of ester and the percentage of the total

TABLE 1.

Micrograms of cholesterol (ester or free) present in rat sternal lymph nodes at intervals following the intraperitoneal injection of cholesterol suspensions mixed with control, corn oil or coconut oil emulsions. (Mean of 8 observations together with the estimated standard error.)

Preparation injected	Cholesterol fraction	24 hours	4 days	10 days
Cholesterol with albumin and control "emulsion"	Ester	107 ± 9·6	114 ±13·1	10 ± 2·9
	Free	577 ±55·0	234 ±16·3	192 ±16·4
	p.c. Ester	15·9± 1·02	32·3± 1·93	5·7± 1·7
Cholesterol with albumin and corn oil emulsion	Ester	158 ±13·7	232 ±31·4	47 ± 7·7
	Free	824 ±74·5	304 ±21·4	239 ±15·7
	p.c. Ester	16·2± 0·65	42·3± 2·21	16·2± 2·4
Cholesterol with albumin and coconut oil emulsion	Ester	112 ± 7·5	240 ±35·2	81 ±14·2
	Free	778 ±86·6	345 ±18·0	230 ±11·5
	p.c. Ester	13·2± 1·04	39·8± 2·48	25·1± 2·7

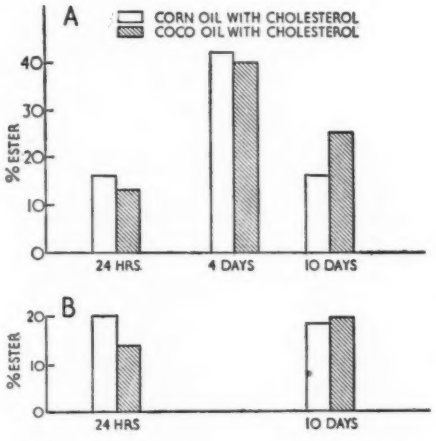

Fig. 1. Percentage of the total cholesterol present as ester in sternal lymph nodes at intervals following the intraperitoneal injection of cholesterol with either corn oil or coconut oil.
A. Single injection of corn or coconut oil.
B. Repeated injection of corn or coconut oil.

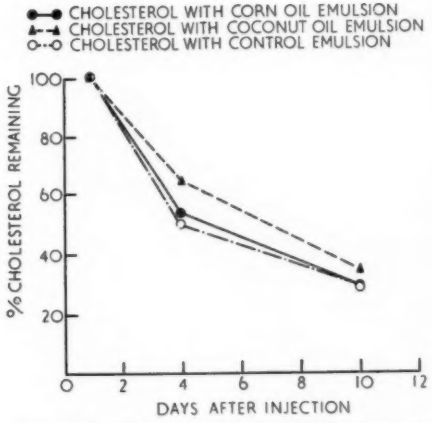

Fig. 2. Removal of cholesterol from the sternal lymph nodes. The total cholesterol present at intervals after the injection of cholesterol, cholesterol with corn oil and cholesterol with coconut oil is expressed as the percentage of that present at 24 hrs.

cholesterol present as ester had risen, while the free cholesterol had been reduced during this time. There was, at 4 days, no apparent difference between the percentage ester present in the corn oil and coconut oil groups, but in both groups significantly more ester was present than in the control ($p < 0{\cdot}01$). By 10 days, during which time the free and ester cholesterol had fallen in the nodes, the percentage ester present in the coconut oil group was significantly

higher than that in the corn oil group ($p < 0{\cdot}05$), while in the control practically no ester remained.

When the removal rate of total cholesterol from the nodes was examined, no sustained difference between the three groups could be detected. Cholesterol injected with corn oil emulsion was removed from the nodes at the same rate as cholesterol injected with the control emulsion (Fig. 2). There was some initial delay in removal in the presence of coconut oil, but the removal rate over the 4-10-day period was similar to that in the other two groups.

TABLE 2.

The effect of repeated doses of corn oil and of coconut oil on the free and ester cholesterol content of rat sternal lymph nodes following the uptake of cholesterol. Micrograms of cholesterol (ester or free) present in the nodes at intervals after the initial injection of cholesterol and emulsion. (Mean of 8 observations together with the standard error.)

Experiment	Cholesterol fraction	24 hours	10 days
Cholesterol with albumin and corn oil emulsion	Ester	*259 ±22·0	64 ± 8·3
	Free	1008 ±72·4	278 ±20·8
	p.c. Ester	20·5± 0·80	18·8± 2·27
Cholesterol with albumin and coconut oil emulsion	Ester	141 ±20·8	53 ±12·2
	Free	833 ±63·5	213 ±23·6
	p.c. Ester	14·2± 0·99	20·0± 3·40

* Seven rats only.

In the second experiment (Table 2), it was again found that the percentage ester formed within 24 hours was significantly less in the presence of coconut oil than in the presence of corn oil ($p < 0{\cdot}001$). Fig. 1B shows the percentage ester present in the nodes at 24 hours and at 10 days. By 10 days the initial

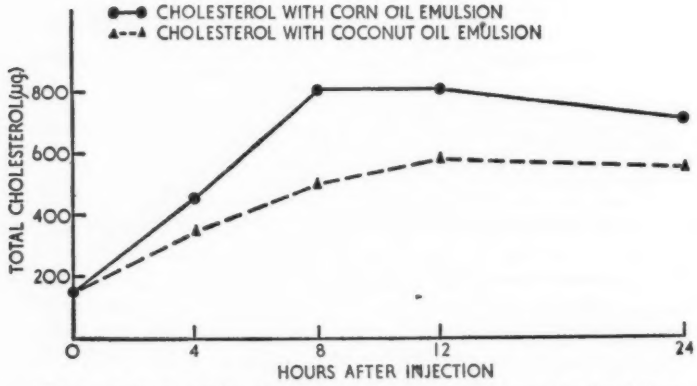

Fig. 3. Rate of uptake of cholesterol by the sternal lymph nodes following the intraperitoneal injection of cholesterol with either corn oil or coconut oil. Total cholesterol in the nodes at intervals during the first 24 hours after injection is shown.

TABLE 3.

Micrograms of cholesterol (ester or free) in sternal lymph nodes at intervals during the first 24 hours after intraperitoneal injection of cholesterol suspensions with either corn oil or coconut oil emulsions.

Experiment	Cholesterol fraction	4 hours	8 hours	12 hours	24 hours
Cholesterol with albumin and corn oil emulsion	Ester	7 ± 7·0	34 ± 7·4	72 ± 10·4	137 ± 9·2
	Free	450 ±68	773 ±157	729 ±121	575 ±63
	p.c. Ester	1·1± 1·1	4·2± 0·5	9·1± 0·4	19·5± 0·9
Cholesterol with albumin and coconut oil emulsion	Ester	0	12 ± 1·0	37 ± 5·3	112 ±20·2
	Free	346 ±49	486 ± 51	545 ± 45·8	445 ±63
	p.c. Ester	0	2·5± 0·3	6·5± 1·0	19·9± 2·7

difference in percentage ester had been reversed, and there was a higher proportion of cholesterol present as ester in the coconut oil group. The removal rate of total cholesterol from the nodes was similar over the 10-day period for the two groups.

The uptake of cholesterol by the nodes during the first 24 hours (Exp. 3) in the presence of either corn oil or coconut oil, is shown in Fig. 3. The ester, free cholesterol and the percentage ester present in the nodes at intervals during the first 24 hours are shown in Table 3. The percentage of the cholesterol present as ester was significantly less in the presence of coconut oil than in the presence of corn oil ($p < 0{\cdot}05$ at both 8 and 12 hours). The uptake of cholesterol with corn oil, however, follows the same pattern as with coconut oil. Although the maximal amount of cholesterol taken up by the nodes was less with coconut oil, the absorption peak occurred in both groups at approximately 12 hours.

DISCUSSION.

The removal rate of cholesterol taken up by the reticulo-endothelial cells of the nodes was unaffected by the presence of either corn oil or coconut oil. Since esterification of cholesterol occurs in the nodes (Day and French, 1959) it is possible that the cholesterol would be esterified with polyunsaturated fatty acids in the presence of corn oil, and with saturated fatty acids in the presence of coconut oil. Triglycerides can be hydrolysed by reticulo-endothelial cells (Day and Harris, 1960) so that fatty acids from the oil emulsions would be available during the esterification process. However, triglycerides are removed from the nodes within 2-3 days (French and Morris, 1960); it is therefore possible that the similar removal rate in Experiment 1 might be explained by esterification after 2-3 days occurring with endogenous fatty acids as in the control group. The repeated injection of corn oil or of coconut oil during the period of cholesterol removal, however, also failed to produce any difference in the rate of removal of cholesterol. The assumption has been made that cholesterol is esterified with polyunsaturated fatty acids in the presence of corn

oil and with saturated fatty acids in the presence of coconut oil. If this is valid, it can be concluded that esterification of cholesterol with polyunsaturated fatty acids rather than with saturated fatty acids does not facilitate its removal from reticulo-endothelial cells.

There were differences, however, in the rate of accumulation and removal of cholesteryl esters in the nodes following the injection of cholesterol with either corn oil or coconut oil. The percentage of the cholesterol present as ester in the first 24 hours was less with coconut oil than with corn oil. During the subsequent removal of the cholesterol from the nodes the percentage present as ester rose more rapidly in the presence of coconut oil than it did in the presence of corn oil. The percentage ester present in the nodes at any one time is influenced by several variables, such as the peak of maximal absorption of cholesterol, the rate of formation of ester and the rate of removal of ester. The peak of maximal absorption, however, was approximately the same when the cholesterol was mixed with coconut oil or with corn oil. Portman and Sinisterra (1957) have demonstrated that ^{14}C labelled cholesterol is esterified at a slower rate in animals fed a diet rich in saturated fatty acids. It is possible that the reduced accumulation of cholesteryl ester which occurs in the nodes in the presence of coconut oil may be due to slower esterification of the cholesterol with the saturated fatty acids. The rising percentage of such esters during the 10-day period may likewise indicate a slower hydrolysis of the saturated fatty acid esters of cholesterol compared with polyunsaturated fatty acid esters.

Acknowledgments. I wish to thank Professor Sir Howard Florey for his continued interest in this work and Drs. J. E. French, D. S. Robinson and J. C. F. Poole for their advice and encouragement. I am indebted to Mr. G. J. Wren for technical assistance and to the Medical Research Council of Great Britain for financial assistance.

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A CLOSED METHOD FOR THE MICROSCOPIC STUDY OF THE LIVING RAT PANCREAS *IN SITU*

by A. A. PALMER AND A. G. WILKINS

(From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney).

(*Accepted for publication 26th July, 1960.*)

SUMMARY.

A method is described for the microscopic observation of the pancreas of the rat which affords clear vision for periods up to 6 hours with minimal damage to the tissues and does not require the use of irrigating solutions.

INTRODUCTION.

The living rodent pancreas has long been an object for microscopic observation. Because of its thinness and mobility and the transparency of its peritoneal covering, the living acinar cells and blood vessels can be clearly seen. The rabbit's pancreas has been extensively studied (Kühne and Lea, 1882; Matthews, 1899; Natus, 1910; Ricker and Regendanz, 1921; Nordmann, 1933; Illig, 1955). Their technique involved withdrawing the duodenum with its mesentery in which the head of the pancreas lies and irrigating the latter with physiological saline at body temperature; a coverslip was used by Kühne and Lea, but discarded by Natus and most subsequent investigators. Covell (1928) and Berg (1930) used the tail of the pancreas in the white mouse; the spleen and tail of the pancreas were delivered through a wound, bathed in physiological saline at body temperature and covered with a coverslip. Flory and Thal (1947) described a transparent chamber for the study of the tail of the pancreas in the living mouse; the spleen was removed and the tail of the pancreas placed in a lucite chamber projecting from the back of the mouse.

Methods similar to all these have been tried in the rat with some success; however, many of the animals were unsuitable because of the shortness of the duodenal mesentery or pancreatic tail, or because of adhesions. Movement with respiration and peristalsis, and inaccessibility to high power lenses were further problems. It was also difficult to keep the rat warm and dry. The method to be described was developed to overcome these difficulties during a study of blood flow in minute vessels of the pancreatic region of the rat (Palmer, 1959).

METHOD.

The apparatus. The principle of the apparatus is indicated in Fig. 1, which is a diagrammatic cross section of the abdomen of the rat with the apparatus in position. The objective lens of the microscope operates in a well with a coverslip sealed in its floor. The well passes through the anterior abdominal wall with an airtight fit. A perspex rod introduced through a separate flank incision supports and illuminates the pancreas. Little evaporation can occur and irrigating fluid is not needed. Fig. 2 shows the equipment in use. Fig. 3 is an exploded view. Details of construction are shown in Fig. 4.

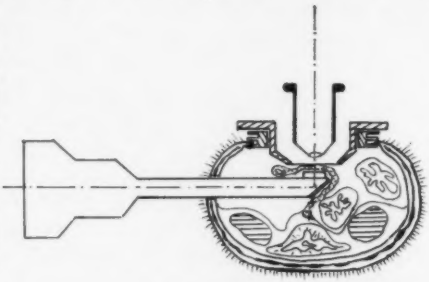

Fig. 1. *Diagrammatic cross section of the abdomen of a rat with the apparatus in position.* (After Palmer, 1959.)

The numerals in the following description apply to Fig. 3. There are two essential components.

(A) A rigidly supported perspex well (8) with a coverslip sealed into its floor. This projects into the rat through a precisely machined ring (7), which, in turn, fits closely into the anterior abdominal opening. The well allows the objective lens to be brought near the pancreas. The perspex ring (7) has two grooves on its outer surface; the upper groove permits the ring to be aligned and held firmly by three screws tapped horizontally into a brass plate (6), which is supported by four studs from the base-plate (1), so that it lies horizontally just above the abdomen of the rat; into the lower groove in the perspex ring the edges of the abdominal wound fit fairly tightly, much as a tyre fits on the rim of a wheel.

(B) A perspex rod (4) which conveys the light and serves as a table for the pancreas. This rod is rigid, but can be moved vertically with a rack and pinion made from the condenser mounting of an old microscope. One end of the rod is bevelled at 45° to reflect the light at right angles. The bevelled end is protected from contact with wet tissues by a plastic coverslip which is sealed to the end around its edges so as to enclose a small air gap; this ensures total reflection. The microscope condenser, with top lens removed to increase the focal length, is mounted close to the other end of the perspex rod in a sleeve (5).

A high pressure mercury vapour lamp was used as a source of light because the low emission in the infra-red causes

Fig. 2. *Photograph of the equipment in use.* (Reproduced by courtesy of the Quarterly Journal of Experimental Physiology.)

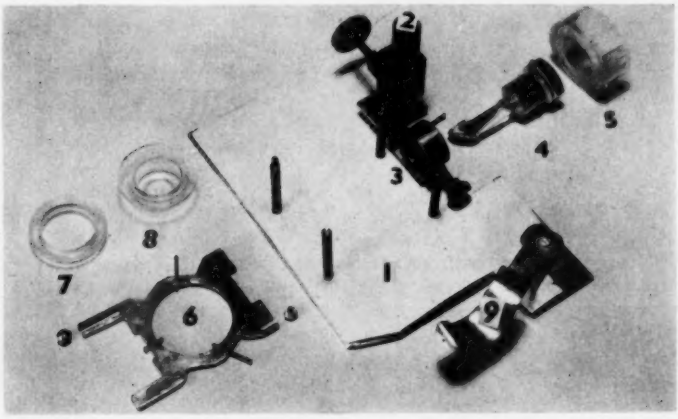

Fig. 3. *Exploded view of the apparatus.*

1. Duralumin base-plate with celluloid cover.
2. Rack and pinion.
3. Brass holder for perspex rod. There is a groove on the inner surface of the brass ring which locates a projecting screw on the back of the perspex rod so that the flat surface on which the pancreas rests is kept in a horizontal plane, parallel with the coverslip.
4. Perspex rod to convey light and act as support for the pancreas.
5. Housing for microscope condenser. This fits on to the hub of the perspex rod.
6. Brass plate which supports well.
7. Perspex ring which holds well.
8. Perspex well with glass or plastic coverslip in floor.

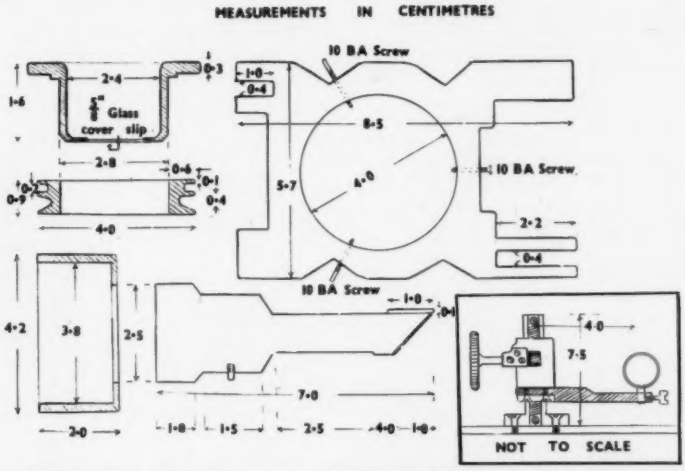

Fig. 4. *Details of components.* Measurements are in centimetres.

little heating even with strong illumination; moreover, the red cells appear in sharper contrast with this bluish light. A filter was used to remove ultra-violet radiation. For photography an electronic flash was generally necessary. The microscope was a Reichert binocular-eyepiece model which has the advantage of a good clearance between the bottom end of the oil-immersion objective and the stage. The objective carrier and binocular body on this microscope both slide in a groove on the front of the coarse adjustment rack, and the clamps which lock them are still effective when they are raised about 1·5 cm. above the normal position; this gives a maximum clearance of about 5·7 cm. and permits rotation of the nosepiece from one objective to another simply by racking up the coarse adjustment, even when the apparatus is made to accommodate a large rat. A microscope with less clearance could be used with smaller rats.

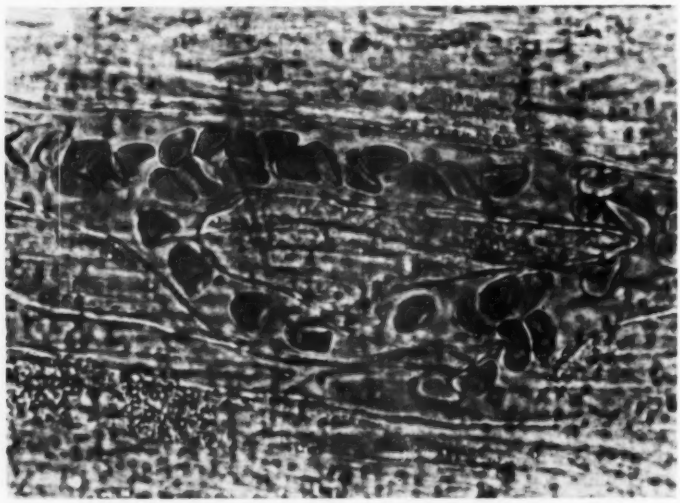

Fig. 5. *Capillary network in transparent mesentery between pancreatic lobules.* Electronic flash and oil-immersion objective were used. The very short exposure time of the electronic flash gives a clear image of the moving red cells which are distorted in shape by the stresses due to flow.

Method of examination. The rat was first anaesthetised with ether followed by nembutal, 4 mg./100 gm. body weight, injected intramuscularly into the thigh. Veterinary nembutal was used, diluted 1 in 8; half the initial dose was repeated as required. An upper midline abdominal incision was made. This was done by pinching up the skin with forceps and cutting off the resulting fold with scissors; the defect in the skin was almost circular. Any bleeding, which was usually slight, was arrested before opening the peritoneum. A cut was made along the linea alba with scissors since this caused minimum bleeding. This cut was made a little longer than the diameter of the skin wound, to fit the lower groove in the perspex ring. An opening was next made in the flank for the illuminating rod, a small fold of skin was cut off with scissors as before, then a light was held near the skin opening and a stab wound with a scalpel was made from within out. In this way the large vessels could be seen and avoided.

The rat was then placed in position on the tray, the perspex ring was fitted into the anterior incision, the illuminating rod was passed into the flank wound and the ring and rod were clamped in their respective holders. The microscope condenser with top lens removed was slipped into the sleeve at the outer end of the illuminating rod. A light was held near the outer end of the rod to illuminate the field for the remaining procedure. The rod was elevated and the duodenum was draped over the rod with a selected region of pancreas on the table. If the peritoneal fluid had become contaminated with blood, the surface of the pancreas was washed gently with physiological saline or Ringer's solution at 38° C.

The well was then inserted so that the coverslip just touched the most projecting part of the pancreas and the rod was racked down while the well was simultaneously lowered in the ring. This manoeuvre prevented other viscera from encroaching on the table. The apparatus was then placed on the microscope stage, the light was aligned with the condenser and the final adjustment of the rack and pinion made under direct vision with a very low power (X 4 lens). By observing veins near the edge of the table, undue compression of the pancreas could be avoided at the same time securing a thin, flat, almost stationary field.

RESULTS.

The following structures could be clearly seen: pancreatic acini, islets of Langerhans, pancreatic ducts, the common bile duct, contractile lacteals, slender nerves and islands of adipose tissue. The blood vessels of all these structures could be seen with varying clarity. Those accompanying lacteals and nerves in the transparent duodenal mesentery were particularly favourable for critical study; their walls and contents could be seen more clearly than, for example, those of the vessels in the frequently studied rat meso-appendix where the connective tissue is less transparent. In favourable vessels platelets could be clearly seen.

The method has the following advantages:

1. The method is applicable to rats and the technique is simple and quick, the entire procedure taking only five to ten minutes.
2. Damage to the tissue by exposure and handling is minimised because the pancreas can be rapidly arranged in position without being touched and without tension in the mesentery.
3. It is easy to move a different part of the pancreas into the field of vision by momentarily withdrawing the well.
4. The pancreas is in its natural environment except for contact with the perspex rod and glass coverslip.
5. All objective lenses including high dry and oil-immersion can be used without fogging.
6. A rack and pinion permits delicate adjustment of the gap between table and coverslip so that the pancreas is gently flattened without damage and a flat, thin, bubble-free, stationary field is available.
7. The technique can be easily modified for studying other abdominal organs.
8. A standard microscope can be used with only minor adjustments.

Temperature control. With a room temperature of 23° C. thermocouple measurements showed that the upper surface of the pancreas remained at 30° C. There was little difference in the circulation or behaviour of the vessels at this temperature even after 6 hours. Thus for many observations heating is unnecessary. For some experiments the temperature of the part of the pancreas under observation was maintained at 38° C. by directing a stream of electrically-heated air into the well.

Local application of drugs. By using a well with a thicker perspex wall it was possible to insert a fine polythene tube through a vertical canal in the wall of the well. The canal was made to turn obliquely near its lower end and thus direct the polythene tube beneath the coverslip. Substances could then be injected through the tube on to the region under observation.

The size and shape of the object table. For studying the thinnest parts of the mesentery between the pancreatic lobules a small table 5 mm. x 3 mm. was used. A small table was also found more suitable for the meso-appendix.

Acknowledgments. We should like to thank Professor Sir Roy Cameron, F.R.S., for his advice and encouragement and Mr. R. A. Money for assistance with the photography. This work was commenced in the Department of Morbid Anatomy, University College Hospital, London.

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THE REMOVAL OF UNESTERIFIED FATTY ACIDS BOUND TO ALBUMIN FROM THE PERITONEAL CAVITY OF THE RAT

by A. K. LASCELLES AND BEDE MORRIS

(From the Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra).

(*Accepted for publication 30th September, 1960.*)

SUMMARY.

The absorption of T-1824 labelled albumin and ^{14}C labelled unesterified fatty acid from the peritoneal cavity of the rat has been studied. It was found that labelled fatty acids were removed much more rapidly than labelled albumin. While the concentration of both total and labelled albumin in the peritoneal fluid fell progressively during the period of absorption, the concentration of total unesterified fatty acids in the peritoneal fluid rose slightly.

The results suggested that unesterified fatty acids exchanged rapidly in both directions across the peritoneal serous membrane. In the case of albumin, there was a unidirectional transfer of unlabelled protein into the peritoneal cavity. The net removal of both albumin and unesterified fatty acids occurred together with the absorption of fluid through the lymphatic system.

INTRODUCTION.

Unesterified fatty acids are transported in the blood bound for the most part to albumin (*cf.* Fredrickson and Gordon, 1958). It has been shown (Havel and Fredrickson, 1956; Laurell, 1957) that unesterified fatty acids have a circulating half-life of only a few minutes compared with several hours for plasma albumin. This suggests that the albumin-fatty acid complex dissociates and that the fatty acid moiety leaves the circulation independently of the albumin.

The structure of the capillary membrane, through which the fatty acids must pass, varies throughout the body. The amount of fatty acid removed by a tissue is probably related in some way to the structure of the capillary membrane and the metabolism of the surrounding tissue cells. The peritoneal cavity is a modified tissue space which is lined by serosal cells which resemble vascular endothelium very closely.

This paper describes the results of an experiment which was designed to establish whether unesterified fatty acids and albumin were absorbed from the peritoneal cavity at the same rate and by the same mechanism.

Materials and Methods.

Preparation of unesterified fatty acid-albumin.

Blood was collected from the abdominal aorta of rats and the serum was separated. 30 μc. of ^{14}C palmitic acid (Radiochemical Centre, Amersham) were evaporated from benzene to dryness under a stream of nitrogen and saponified with a slight excess of 0·01 N. NaOH solution at 70° C. The serum (110 ml.) was added to the soap solution and mixed thoroughly. Prepared in this way, about 95 p.c. of the radioactivity was bound to albumin.

The albumin was labelled with Evans blue (T-1824). The dye was dissolved in rat serum to give a final concentration of 20 mg. of T-1824 in 100 ml. of serum. This concentration was well below the theoretical binding capacity of albumin for T-1824 (Rawson, 1943; Allen and Orahovats, 1950) and a minimum of free dye anions was present in the solution.

Experimental procedure.

Twenty male rats weighing between 190 gm. and 200 gm. were divided randomly into 5 groups of 4 rats. Each rat was injected intraperitoneally with 5 ml. of the labelled serum (Morris, 1956). The 4 rats composing a group were killed with chloroform at ¼, 1, 2, 4 and 8 hrs. after the injection and the fluid within the peritoneal cavity was recovered with a Pasteur pipette and the volume measured.

The following measurements were made on the injected serum and the recovered fluid:

(1) The concentration of T-1824 was measured by extraction and absorption onto cellulose columns using the method of Allen (1951). Optical densities of the acetone-water extracts were measured at 625 mμ with a Beckman Model DU spectrophotometer.

(2) The radioactivity of the fluids was measured by plating duplicate 0·1 ml. samples onto polythene planchets to which 0·1 ml. of 5 p.c. trichloracetic acid had been added. The samples were covered with lens tissues discs, evaporated to dryness and counted with a thin mica end-window tube to an accuracy of ± 2 p.c.

(3) Unesterified fatty acids were measured by a modification of the method of Dole (1956). Instead of 1 ml. of serum, 0·5 ml. was extracted into heptane and 1 ml. of this heptane phase was used for titration. Two titrations were done on each extract and the mean taken. Duplicate determinations agreed within ± 5 p.c.

(4) The proteins in each sample of fluid were separated by paper electrophoresis using a barbiturate buffer, pH 8·6, ionic strength 0·06 M. The patterns were stained with bromophenol blue and the dye in the albumin fraction was eluted and measured spectrophotometrically at a wavelength of 595 mμ. The amount of dye present was used as a measure of the albumin concentration.

RESULTS.

Calculation of results. No fluid was recovered from the peritoneal cavities of rats killed 8 hrs. after injection. The analysis of the results, therefore, has been confined to the ½- to 4-hr. period. As a logarithmic time series was chosen for the experiment, the individual results were expressed as percentages of their ½-hr. values. The regression coefficients relating the percentage remaining in the peritoneal cavity to the logarithm of time were calculated over the ½- to 4-hr. period for each constituent. These regression coefficients were then used to compare the rates at which the various constituents were removed.

The rates of removal of fluid, albumin and fatty acids from the peritoneal cavity.

The removal of fluid, T-1824, albumin and labelled palmitic acid from the peritoneal cavity is shown in Fig. 1. Approximately 90 p.c. of the labelled fatty acids were removed within the first hour compared with 40 p.c. of the labelled albumin and 10 p.c. of the fluid.

The absorption of fluid was linear after the first half hour. The volume of fluid recovered at the half hour interval was approximately the same as the volume injected and this suggested that fluid entered the peritoneal cavity during the first 30 min. at about the same rate as it was absorbed.

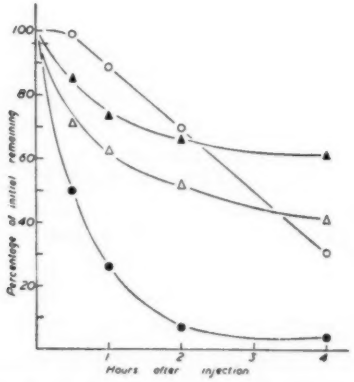

Fig. 1. The removal of ^{14}C labelled palmitic acid, T-1824, albumin and fluid from the peritoneal cavity of the rat following the injection of albumin-bound palmitic acid. The results at each time interval are the mean values for groups of 4 rats.

- ● palmitic acid
- △ T-1824
- ▲ albumin
- ○ fluid.

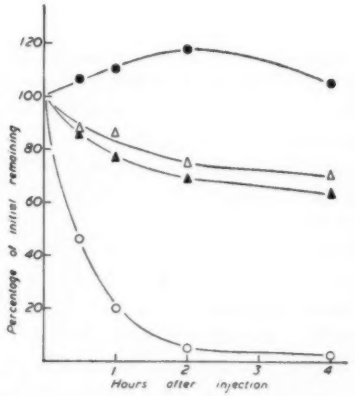

Fig. 2. The changes in concentration and specific activity of unesterified fatty acid and albumin in the peritoneal cavity of the rat following the injection of albumin-bound palmitic acid.

- ● concentration of unesterified fatty acids
- ○ specific activity of unesterified fatty acids
- ▲ concentration of albumin
- △ specific activity of albumin.

The removal of labelled albumin, as measured by the changes in concentration of T-1824 in the recovered fluid, followed an exponential function. The removal of total albumin, both labelled and unlabelled, followed the same general course, but was significantly slower ($P < 0{\cdot}01$). The regression coefficients for T-1824 and total albumin were $-13{\cdot}79 \pm 1{\cdot}0$ and $-8{\cdot}87 \pm 0{\cdot}99$ respectively. The summary of the analyses of variance relating the changes in concentration of T-1824 and total albumin in the peritoneal fluid to time are given in Tables 1 and 2.

TABLE 1.

Summary of the analysis of variance of the changes in T-1824 concentration against log time.

	Source of variation	D.F.	Sum of squares	Mean square
Group means		4	109,050·80	
	Grand mean	1	105,214·30	
	Linear regression	1	3,803·69	3,803·69***
	Remainder	2	32·80	16·40
Variance		11	220·11	
Total		15	109,270·91	

*** $P<0·001$.

TABLE 2.

Summary of the analysis of variance of the changes in albumin concentration against log time.

	Source of variation	D.F.	Sum of squares	Mean square
Group means		4	117,991·60	
	Grand mean	1	116,390·14	
	Linear regression	1	1,573·89	1,573·89***
	Remainder	2	27·60	13·80
Variance		11	214·83	19·53
Total		15	118,206·43	

*** $P<0·001$.

The specific activity of the albumin in the peritoneal cavity fell by about 25 p.c. during the 4-hr. period of absorption. This fall in specific activity was due to the addition of unlabelled albumin to the peritoneal fluid. Most of the unlabelled albumin entered the peritoneal cavity during the first hour following the injection; thereafter the specific activity and albumin concentration curves were almost parallel (Fig. 2).

TABLE 3.

Summary of the analysis of variance of the changes in radioactivity against log time.

	Source of variation	D.F.	Sum of squares	Mean square
Group means		4	50,384·24	
	Grand mean	1	29,338·55	
	Linear regression	1	18,985·73	18,985·73***
	Quadratic regression	1	2,049·82	2,049·82***
	Cubic regression	1	10·13	10·13
Variance		12	626·64	52·22
Total		16	51,010·88	

*** $P<0·001$.

The labelled fatty acids were removed very rapidly; the rate of removal after the first half-hour was expressed by the linear regression coefficient $b = -30 \pm 1{\cdot}62$. This was significantly greater ($P < 0{\cdot}001$) than for T-1824 or total albumin. The analysis of variance for the changes in radioactivity is summarized in Table 3.

The concentration of unesterified fatty acids in the peritoneal fluid rose slightly during the experiment, but this rise was not statistically significant. This meant that, as the labelled fatty acids were removed, unlabelled fatty acids entered the peritoneal cavity and the concentration thus remained unchanged. The specific activity curve for fatty acids followed the curve for radioactivity very closely (Fig. 2).

DISCUSSION.

The results demonstrate clearly that when albumin-bound fatty acids are injected into the peritoneal cavity, the fatty acids are absorbed much more rapidly than the albumin. It is known that albumin is absorbed from the peritoneal cavity almost exclusively by way of the lymphatics of the diaphragm (*cf.* Courtice and Simmonds, 1954). The fact that there was a rapid decrease in radioactivity while the concentration of fatty acids did not change significantly suggested that an equilibrium was reached between the unesterified fatty acids in the peritoneal fluid and the tissue fluid outside the peritoneal cavity. It appeared that a rapid transfer of both labelled and unlabelled fatty acid molecules occurred in both directions across the peritoneal membrane. When an equilibrium was established, the net removal of unesterified fatty acids occurred by means of the bulk transfer of fluid containing the fatty acids, through the lymphatics of the diaphragm. Although the concentration of albumin in the peritoneal fluid decreased throughout the experiment, there was no evidence to suggest that the amounts of fatty acid present exceeded the available binding sites provided by the albumin.

Morris (1956) studied the removal of heparinized fatty chyle from the peritoneal cavity and found that there was no significant difference between the rate of removal of chylomicron triglycerides and albumin. It might be expected that, if the injection of heparin intraperitoneally caused the release of clearing factor lipase into the peritoneal fluid, the chylomicron triglycerides would be hydrolyzed and the fatty acids absorbed rapidly. It was found, however, in these present experiments that only a small amount of lipase activity could be demonstrated in the peritoneal fluid when heparinized plasma was injected intraperitoneally or intravenously.

It is possible to postulate a series of exchange and removal processes which may occur when labelled unesterified fatty acids and albumin are injected into the peritoneal cavity. An equilibrium is probably established between the concentration of albumin in the peritoneal fluid and the sub-peritoneal tissue

fluid over a period of several hours. This is brought about by the transfer of albumin and fluid from the tissue spaces into the peritoneal cavity. Both labelled and unlabelled albumin are removed from the peritoneal cavity through the lymphatics of the diaphragm. With unesterified fatty acids, however, an equilibrium is probably established between the concentration of fatty acids in the sub-peritoneal tissue fluid and the peritoneal cavity within the first hour. Most of the labelled fatty acid molecules would be removed by direct transfer through the general peritoneum. The net removal of unesterified fatty acids would occur with albumin, as fluid and protein are absorbed into the lymphatic system of the diaphragm.

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A HEAT STABLE PROTECTIVE STAPHYLOCOCCAL ANTIGEN

by STEPHEN FISHER

(From the Kanematsu Memorial Institute of Pathology, Sydney Hospital).

(*Accepted for publication 3rd August, 1960.*)

SUMMARY.

A strain of staphylococci, chosen for the very marked enhancement by mucin of its intraperitoneal virulence to mice, was cultured in sloppy agar.

The supernatant fluids obtained were of low toxicity and were highly potent as active immunizing agents in mice against lethal intraperitoneal challenge by the homologous strain in mucin suspension. The activity of the protective antigen was not affected by boiling for 10 minutes and was retained in the bag during dialysis.

The protection could be transferred passively by means of the sera of immunized animals.

INTRODUCTION.

Strains of staphylococci which appear to be pathogenic to man kill mice in smaller doses by the intraperitoneal route if the bacteria are suspended in mucin than if they are administered in saline (Schneierson and Amsterdam, 1957). This enhancement of virulence by mucin is particularly marked with certain strains. One such strain was used in the work described below; culture fluids were prepared and experiments carried out on their toxic and immunizing properties.

MATERIALS AND METHODS.

Strain. Staphylococcus strain 90B of this laboratory was the only one used. It was obtained as Strain Smith from Dr. M. W. Fisher, Parke Davis and Co., Detroit, U.S.A. In this laboratory, it was slightly haemolytic on human blood and sheep blood agar, and produced a moderate amount of golden pigment; coagulase was demonstrated by the tube test, but not by the slide test. The virulence to mice by the intraperitoneal route was greatly enhanced by mucin (Fisher, 1960). In these respects the strain was similar to strain Smith as described (Smith and Dubos, 1956; Finkelstein and Sulkin, 1958; Hunt and Moses, 1958; and M. W. Fisher, 1959). However, when examined by Dr. Phyllis M. Rountree, it was not lysed by any of the phages in the standard staphylococcal typing set. It may be noted that strain Smith has been described as consisting of two variants, one of which is untypable and the other susceptible to lysis by phages 44A and 42E (Finkelstein and Sulkin, 1958; Hunt and Moses, 1958).

Preparation of culture fluids. 100 ml. lots of sloppy agar, consisting of 0·15 p.c. New Zealand agar in Difco heart infusion broth, were dispensed in 16 oz. flat bottles and inocu-

lated with 1 ml. of the bacteria covering an overnight grown nutrient agar slope culture of strain 90B suspended in 10 ml. nutrient broth. Some bottles were plugged with cotton wool. In others the air was replaced with a mixture of 80 p.c. oxygen and 20 p.c. CO_2; these were closed with rubber stoppers. These cultures will be referred to as air incubated and O_2/CO_2 incubated ones, respectively. The bottles were incubated on their sides at 37° C. for 3 days, with occasional shaking. The purity of the cultures was then checked by smear and Gram stain, and the bottles placed in the deep freeze overnight. The contents were thawed, centrifuged at 5° C. and 9000 r.p.m. for 20 minutes and the clear supernatants decanted into two pools, one from the cultures incubated in air and the other from those incubated in O_2/CO_2. Thiomersal was added to 0·01 p.c. and the fluids stored in the refrigerator.

Fluids made in a single batch were used in the experiments described below; similar results were obtained with other batches.

Immunization and challenge of mice. Adult white mice were used. Immunizing injections were given subcutaneously over the back. For active immunization 0·5 ml. culture fluid dilution was injected, for passive immunization, 0·2 ml. serum dilution. Dilutions were made in saline. Unless otherwise stated, the mice were challenged 10 days after active and 24 hours after passive immunization with suspensions prepared by mixing one volume bacterial suspension in saline containing 10^5 organisms per ml. with 10 volumes 5 p.c. mucin. Cultures of strain 90B grown overnight on nutrient agar were used for challenge, the strength of the saline suspensions standardized against Brown's tubidity tubes (Burroughs Wellcome) and further dilutions to 10^5 bacteria per ml. made in saline. The mucin (hog gastric mucin Type 1701W, The Wilson Laboratories, Chicago) was made up according to the manufacturer's instructions. One ml. of the challenge dose was injected intraperitoneally; in the strength described above, this represented 100 LD50's (Fisher, 1960). The mice were observed for one week after challenge.

Estimations of alpha haemolysin and antihaemolysin were performed according to the method of Burnet (1931). In antihaemolysin estimations, the international standard was used as a reference serum.

RESULTS.

Solid content of the O_2/CO_2 incubated fluid. Duplicate 10 ml. samples were dialysed in cellophane bags against running tap water for 24 hours, then against distilled water overnight in the refrigerator, then evaporated on a water bath and finally heated to constant weight at 110° C. The solid content of undialysed fluid was also determined.

The undialysed fluid contained 2·3 p.c. and the dialysed fluid 0·32 p.c. solids (both w/v).

Toxicity tests. (a) In mice. Groups of two mice were injected subcutaneously with 0·5 ml. volumes of air incubated and O_2/CO_2 incubated fluids diluted with saline 1/2·5, 1/5 and 1/10.

One mouse injected with 1/2·5 O_2/CO_2 incubated fluid died the next day. The others were killed after 7 days; the injection sites showed loss of fur and some scarring, more marked in the animals receiving the higher concentrations of fluid. No local or general ill effects were observed in the mice injected with air incubated fluid.

(b) In guinea pigs. Groups of three guinea pigs were given subcutaneous injections of the two fluids, undiluted and diluted 1/5 and 1/25 in saline, in

volumes of 5 ml. per kg. body weight. In the group given O_2/CO_2 incubated fluid, one animal injected with the 1/5 dilution and one receiving the undiluted fluid died; all four survivors of these six animals showed local necrosis and suppuration when killed and examined at 14 days after the injection. The guinea pigs given 1/25 O_2/CO_2 incubated fluid showed scarring at the site of the injection a fortnight after injection. No ill effects were observed following the injection of air incubated fluid.

Alpha haemolysin content. Undiluted air incubated fluid did not haemolyse rabbit erythrocytes completely in 30 minutes at 37° C.; the O_2/CO_2 incubated fluid gave a titre of 1/16.

TABLE 1.
Active immunization of mice.

Number of experiment	Dilution of culture fluid injected	Production of culture fluid	
		In air	In 20 p.c. CO_2 in O_2
		Survivors/total	
1	1/10	10/10	10/10
	Nil (controls)	1/10	
2	1/100	10/10	10/10
	Nil (Controls)	0/10	
3	1/1000 1/5000 1/25,000	8/10 4/10 0/10	10/10 6/10 3/10
	Nil (controls)	1/10	

The mice were immunized subcutaneously with 0·5 ml. culture supernatant dilution and challenged 10 days later intraperitoneally with 1 ml. bacterial suspension in mucin (100 LD50's).

Potency of the fluids as active immunizing agents. In a series of experiments mice were immunized with graded fivefold dilutions of the two fluids. The results, omitting some of the intermediate dilutions up to 1/500, where all the immunized animals survived, are given in Table 1. Protection was complete in the 1/10 and 1/100, and nearly complete in the 1/1000 dilutions, partial in the 1/5000 and slight or absent in the 1/25,000 dilutions. The O_2/CO_2 incubated fluid protected somewhat better than the air incubated one.

Effect of varying the interval between active immunization and challenge (Table 2). From a number of mice set aside for the purpose, groups of 10 were immunized at selected times with 0·5 ml. air incubated fluid diluted 1/50. The

TABLE 2.

Active immunization of mice with challenge after varying intervals.

Interval between immunization and challenge	Survivors/total
5 hours	0/10
24 hours	0/10
2 days	2/10
4 days	10/10
14 days	10/10
28 days	10/10
42 days	9/9
Controls	1/10

The mice were immunized subcutaneously with 0·5 ml. air incubated fluid diluted 1/50. All groups were challenged at the same time intraperitoneally with 1 ml. bacterial suspension in mucin (100 LD50's).

immunized mice and a group of unimmunized controls were challenged at the same time.

All the mice challenged 5 hours and 24 hours, and 8 out of 10 challenged 2 days after immunization died. All the mice challenged at intervals of 4, 14, 28 and 42 days survived. Nine out of 10 controls died.

Passive immunization. A group of mice were injected subcutaneously with 0·5 ml. air incubated fluid diluted 1/50. Fourteen days later half the animals were exsanguinated into a pool; the other half were reinjected and bled out after another 14 days into a second pool. The blood was obtained by severing the axillary vessels under ether anaesthesia. Serum was also collected from a number of unimmunized mice.

A goat, previously used for the production of anti-human globulin serum, was bled and given a series of 8 injections of air incubated fluid diluted in saline containing a total of 12·6 ml. undiluted fluid. The injections covered a period of 4 weeks; the second one was intravenous, the others subcutaneous. A week after the fifth injection and a week after the last injection the animal was bled again.

All sera were separated on the day of collection and stored in the deep freeze.

Antihaemolysin titrations on the above mouse sera, and the first and last specimen from the goat gave the following results:

Serum	Antihaemolysin, Units/ml.
Mouse, unimmunized - - -	0·4
Mouse, 1 x 0·5 ml. 1/50 fluid -	0·4
Mouse, 2 x 0·5 ml. 1/50 fluid -	Less than 0·1
Goat, before immunization -	3
Goat, after immunization (final bleed) - - - - - -	6

TABLE 3.

Passive immunization of mice.

Preparation of immunizing serum		Protection experiments	
Animal	Administration of air incubated culture fluid	Dilution of serum	Survivors/total
Goat	Nil (early bleed)	1/1	0/5
	5 inj., diluted 1/5-1/2, totalling 3·6 ml. undiluted fluid	1/1	0/5
	3 further inj., totalling 9 ml. undiluted fluid	1/1	5/5
		1/10	3/5
		1/100	0/5
Mouse	Nil (normals)	1/1	0/5
	1 × 0·5 ml. 1/50	1/1	5/5
		1/10	2/5
	2 × 0·5 ml. 1/50	1/1	5/5
		1/10	3/5
		1/100	0/5
	Nil (controls)		0/15

Mice were immunized passively with 0·2 ml. serum or serum dilution subcutaneously and challenged 24 hours later with 1 ml. bacterial suspension in mucin (100 LD50's).

In passive immunization tests (Table 3), the goat sera obtained before immunization and after the fifth injection and the pooled normal mouse serum did not protect the recipient animals. The three other immune sera behaved alike. Undiluted, they protected all the animals; diluted 1/10, about half the mice. The serum of the mice immunized with a single injection was not tested in a dilution of 1/100; in this dilution, the other two sera failed to protect the animals.

Active immunization and challenge with larger doses in mucin, or with suspensions in saline. The mice were immunized with 1/100 air incubated fluid. Ten immunized animals and 10 controls were challenged with 1 ml saline suspension containing 10^9 bacteria. Ten immunized and 10 control mice were challenged in the usual way (1 ml. mucin suspension containing 10^4 bacteria); 2 groups of 10 immunized animals each were challenged with 1 ml. mucin suspension containing $10^{6.5}$ and 10^9 bacteria per dose, respectively.

The results are shown in Table 4. The saline suspension, known from unpublished experiments to contain about 20 LD50's, killed all immunized and control mice. The bacterial suspensions in mucin, at the usual challenge level (10^2 LD50's, Fisher, 1960) killed all the controls but none of the immunized mice. Immunized mice challenged with 310 times the usual number of bacteria suspended in mucin ($10^{4.5}$ LD50's) were still significantly protected when compared with the controls (6 survivors out of 9, as against 0 out of 10, $\chi^2 = 6{\cdot}90$,

TABLE 4.

Active immunization of mice; variation of challenge doses.

Challenge dose			Survivors/total	
Suspending agent	Number of bacteria per dose	Number of LD50's per dose	Immunized	Controls
Saline	10^9	2×10	0/10	0/10
Mucin	10^9	10^7	0/10	—
	$10^{8.5}$	$10^{4.5}$	6/9	—
	10^8	10^2	10/10	0/10

The mice were immunized subcutaneously with 0·5 ml. air incubated culture fluid diluted 1/100, and challenged 10 days later intraperitoneally with 1 ml. bacterial suspension.

$p < 0{\cdot}01$), but there was no protection against mucin suspended challenge containing the same number of bacteria as the saline challenge (10^7 LD50's).

Active immunization with heated and dialysed culture fluids. Groups of mice were immunized with 1/10, 1/100, 1/1000 and 1/10,000 dilutions of O_2/CO_2 incubated fluid heated in a boiling water bath for ten minutes; further groups with similar dilutions of the same fluid dialysed against running tap water for 24 hours and then against distilled water overnight in the refrigerator. Two groups were immunized with 1/1000 and 1/10,000 dilutions of the untreated fluid.

TABLE 5.

Active immunization of mice with boiled or dialysed O_2/CO_2 incubated culture fluid.

Treatment of culture fluid	Dilution	Survivors/total
Boiling*	1/10	10/10
	1/100	8/9
	1/1000	10/10
	1/10,000	5/10
Dialysis*	1/10	10/10
	1/100	10/10
	1/1000	9/10
	1/10,000	2/10
Nil (untreated fluid)	1/1000	10/10
	1/10,000	3/10
Unimmunized mice (controls)		0/10

* For details, see Text.

The mice were immunized subcutaneously with 0·5 ml. culture fluid dilution and challenged 10 days later intraperitoneally with 1 ml. bacterial suspension in mucin (100 LD50's).

The results (Table 5) show that neither the heating nor the dialysis caused any appreciable fall in the protective power of the culture fluid.

DISCUSSION.

Some human sera have been found to protect mice against experimental challenge with staphylococci, and sera from patients with boils appeared to be less potent in this respect than other sera (Fisher, 1959). A coagulase-producing strain of staphylococcus aureus was used and the mice were challenged intraperitoneally with living bacteria suspended in mucin; it was known that mucin enhanced the virulence of this strain to an unusual degree.

In the experiments reported in this paper, culture fluids were prepared from the same strain. They contained an antigen which, in amounts of less than 1 μg., protected mice against 100 LD50's of the homologous bacteria injected intraperitoneally in mucin suspension. The antigen withstood boiling and was non-dialysable. Immunity could be transferred to normal mice by the serum of immunized mice; the latter contained so little alpha anti-haemolysin that the protection could not have been due to it. Immunity could also be transferred by the serum of an immunized goat, but a prolonged course of injections was required by the animal before its serum became protective.

Active immunity in mice developed in a few days and lasted for at least 6 weeks. This finding and the transferable nature of the protection by immune serum, suggest that the immunity was due to antibody, and not to specific interference of the type described recently by North and Pawlyszyn (1960).

Although according to the results of the bacteriophage typing, the strain used in this work may not have been strain Smith, the protective antigen described may be identical with the factor isolated from strain Smith supernatant fluids by Morse (1960). Morse found his preparation non-antigenic; this may have been due to degradation during purification. The purified factor inhibited the power of specific rabbit immune serum to neutralise an anti-phagocytic substance produced by this strain.

Studies on the specificity of the protective antigen are in progress.

Acknowledgments. I am indebted to Professor H. R. Carne for arranging for the immunization of the goat; to Mr. Ray Jones for bleeding and injecting the animal; and to Dr. Phyllis M. Rountree for the bacteriophage typing. Technical assistance was provided for under a grant from the Postgraduate Medical Foundation, University of Sydney.

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FACTORS AFFECTING THE STAINING PROCESS IN THE QUANTITATIVE ESTIMATION OF SERUM PROTEINS BY PAPER ELECTROPHORESIS

by GWENDOLINE K. WILKINSON[1] AND G. N. WILKINSON

(From the Institute of Medical and Veterinary Science, Adelaide, and the Division of Mathematical Statistics, C.S.I.R.O., University of Adelaide, South Australia).

(*Accepted for publication 23rd August, 1960.*)

SUMMARY.

A procedure for routine analysis of serum proteins is described, which is virtually free from factors requiring critical control in the staining process, and gives a stable linear dye-protein relationship over a wide range of protein densities.

Optimal staining is achieved only if the concentration of the dye solution and the staining time both exceed inter-related lower limits which in combination depend on the maximum density of protein on paper for which optimal staining conditions are required. Staining for 10 minutes in a 1 p.c. solution of bromphenol blue produces optimal staining for protein densities up to 0·15 mg./cm.^{2}

Freedom from factors requiring critical control is a consequence of optimal staining conditions. A number of staining methods described in the literature are sub-optimal and in consequence require rigid standardization to obtain reproducible results.

INTRODUCTION.

The fractionation and estimation of serum proteins by paper electrophoresis is now a widely used technique. The basic method of estimation is to stain the protein fractions on the paper and then either to elute the dye bound to each fraction or to use a photometric scanning technique, the elution method being generally the more accurate.

There is considerable variation in the literature in regard to the staining methods employed, some of which are evidently sub-optimal. A number of authors, e.g. Franglen and Martin (1954), Hardwicke (1954), Henry, Golub and Sobel (1957), have reported difficulties in obtaining a linear relationship between dye uptake and protein concentration at higher protein levels, and generally it is found that a number of factors affecting dye uptake require critical control.

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[1] Present address: c/o Division of Mathematical Statistics, C.S.I.R.O., University of Adelaide, Adelaide, South Australia.

Franglen and Martin, in particular, have expressed grave doubts on the value of quantitative analysis produced by existing staining techniques.

This paper reports the results of an investigation of factors affecting the staining process with bromphenol blue as the staining agent, and describes the procedure used in this laboratory for routine analysis of serum proteins. This procedure is relatively free from factors requiring critical control in the staining process and gives a stable linear dye-protein relationship over a wide range of protein concentrations.

METHODS.

Routine method for quantitative analysis of serum proteins.

Apparatus. The electrophoretic apparatus designed by Flynn and de Mayo (1951) is used in this laboratory. The apparatus accommodates up to nine paper strips measuring 36 x 4 cm., which are suspended over a central rod with the ends of each paper dipping into the electrode compartments. Whatman 3MM paper is used, with "Veronal" buffer solution (1·84 gm. diethyl barbituric acid, 10·3 gm. sodium diethyl barbiturate per litre of distilled water; pH 8·5) as the conducting agent. Runs of 16 hours duration at room temperature, and at a potential difference of 180 volts (approximately 4 volts/cm.) produce a 12-13 cm. displacement of the albumin fraction towards the anode.

Standards. Six standard solutions of purified human albumin and γ-globulin are used for calibration: 2, 4, 6 p.c. (w/v) of albumin, and 0·5, 1·0, 1·5 p.c. (w/v) of γ-globulin.

Preparation of papers. Usually six sera are analysed in one run, together with the albumin standards. (The globulin standards are used in calibrating the staining process, but are not subjected to electrophoresis.) An amount of 20 µl. of serum or albumin standard is run from a micro-pipette onto each paper strip, in a narrow band 2·5 cm. long located transversely at the midpoint of the strip. Each paper strip is immediately saturated with the buffer solution to within 1 cm. of the protein band, across which the buffer then rapidly diffuses, and the strip thus prepared is mounted in position in the electrophoretic apparatus. This method of applying the buffer solution prevents the band of serum from diffusing outwards appreciably and at the same time gives sufficiently rapid wetting to prevent any denaturation.

Drying and fixing. At the completion of the run the papers are dried in a forced-draught type oven at 80-100° C. At this stage three globulin standards are prepared by pipetting 20 µl. of the standard solutions onto 3 x 4 cm. papers moistened with buffer solution, which are then dried with the others. After approximately 30 minutes drying the papers are immersed for a few minutes in a 10 p.c. (w/v) solution of $HgCl_2$ in ethanol (95 p.c.). The papers are redried and immersed in a water bath at room temperature for 2-3 minutes to remove the sodium barbiturate, which interferes with the staining process. The papers are then dried again.

Staining. The papers are immersed in a 1-2 litre solution of 1 p.c. (w/v) bromphenol blue and 1 p.c. (v/v) acetic acid in ethanol (95 p.c.), for a minimum of 10 minutes. Optimal staining is achieved within this time. The dye solution may be used repeatedly until the calibration curves fall off and become non-linear, indicating the necessity for a fresh solution. If electrophoresis is carried out under conditions which give more dense protein bands, it may be found that the calibration is not completely linear with 1 p.c. bromphenol blue (Hippsley, personal communication), in which case the concentration should be increased to 1·5 or 2 p.c. Hippsley finds, also, that the use of the dye solution can be prolonged by restoring the concentration to the original level from time to time.

Washing. The papers are transferred from the staining bath to a washing solution of 0·5 p.c. (v/v) acetic acid in distilled water. Washing is continued, with regular changes, until the dye bound to the protein has attained a stable green colour, the period of time required being approximately 1½ hours. Initially the washing solution is changed every 10 minutes, but once the papers are fairly free from excess dye, less frequent changes suffice. The papers are then dried.

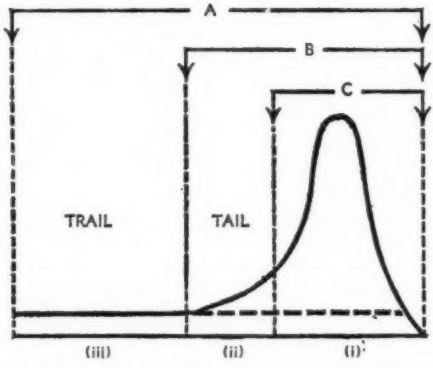

Fig. 1. Schematic diagram illustrating the three sections (i, ii, iii) into which the standard albumin papers are cut. The letters A, B, C refer to the calibration lines in Fig. 2(b).

Cutting for elution. The serum papers are cut into 5 measured sections containing respectively the albumin fraction and the α_1, α_2, β and γ-globulin fractions. It will usually be possible, and very desirable so far as the α_1-globulin and the albumin fractions are concerned, to cut all the serum papers in each run at the same point. It is necessary to record the length of each section containing a globulin fraction so that a correction may be applied for a continuous trail of albumin from the origin.

The standard albumin papers are cut into 3 sections, illustrated in Fig. 1:

(i) the albumin band (the cuts being made as for the serum papers);

(ii) a section (the tail) behind the albumin band corresponding to the α_1-globulin section of the serum papers;

(iii) the remaining portion containing the albumin trail to the point of application.

Usually the albumin band will migrate a slightly greater distance on the standard than on the serum papers. Accordingly, the sections (i) and (ii) above are cut at a greater distance from the origin. Compensation for this difference is effected by cutting a strip off the "trail" equal in length to the extra distance involved, and including this strip with the main albumin band when eluting.

To provide a blank correction, a 3 x 4 cm. section free from protein is cut from one of the papers.

Elution. The dye is eluted from each section with 20 ml. of a solution of 3 p.c. (w/v) Na_2CO_3 in 50 p.c. (v/v) aqueous methanol. The bromphenol blue colour is stable in this eluant. It will be necessary in the case of the albumin bands, or with pathologically high γ-globulin levels, to make a 2- or 3-fold dilution of the eluate to ensure that colorimetric determinations can be made in the more sensitive middle region of the instrument scale (see Fig. 4).

Colorimetric determinations. Determinations are made at the optimum wavelength 595 mμ., with zero optical density set for distilled water. In this laboratory a Unicam S.P. 400 is used. A calibration curve for bromphenol blue should be determined for the instrument to be used (see Fig. 3), since if Beer's law does not hold in the practical range, a curvilinear relation being obtained between optical density and dye concentration, it will be necessary to convert optical density to the equivalent values of dye concentration by the calibration curve.

Standard lines. Calibration of eluted dye concentration with the albumin and globulin standards gives a series of straight lines which are illustrated in Figs. 2(a) and 2(b). (More

than three points are shown in these figures to demonstrate the linearity of the dye-protein relationship.) In the calibration with γ-globulin standards the dye concentrations are corrected for the blank and the calibration line passes through the origin. In calibrating with albumin standards, however, no corrections for the blank are applied. Four calibration lines for albumin are determined,

Line A: Albumin band + tail + trail,
Line B: Albumin band + tail,
Line C: Albumin band only,
Line D: Albumin tail only.

These are illustrated in Fig. 2(b), with the exception of line D, which is preferably drawn with an expanded scale of dye concentration. Though only lines C and D are required for serum estimations more accurate fitting of the lines is obtained by exploiting the mutual relationship of the four lines. The line A deviates slightly from the origin, by an amount corresponding to the blank correction for all three sections. Line B is parallel to A, as the amount of trail is independent of albumin concentration. Line C diverges from B due to the increasing amount of tail, and intersects B slightly below the abscissa.

Estimations. The serum albumin concentration is determined directly from the calibration line C. The correction for tail corresponding to the albumin concentration is determined from line D (not shown in Fig. 2(b)) and subtracted from the dye concentration for α_1-globulin, which is then converted to protein concentration (in γ-globulin equivalents) on the calibration line for γ-globulin. A correction for albumin trail per unit distance is determined by dividing the total of dye concentration values for the trails of the albumin standards by the total length of the trails. (This correction also acts as a correction for blank.) The dye concentrations for the α_2 and β-globulin fractions are then adjusted for albumin trail and converted to protein concentration (in γ-globulin equivalents). The dye concentration for γ-globulin is corrected for blank only, unless the band overlapped the origin towards the anode, in which case a corresponding correction for albumin trail can be applied.

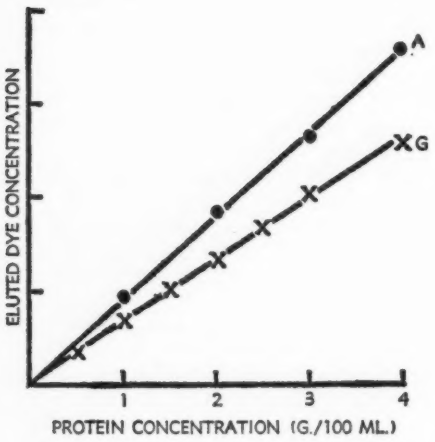

Fig. 2(a). Calibrations of eluted dye concentration (in arbitrary units) against protein concentration. A: albumin. G.: γ-globulin.

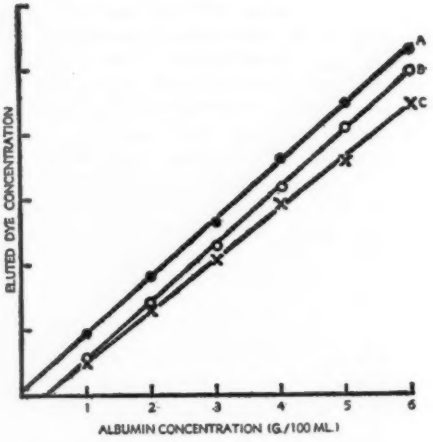

Fig. 2(b). Calibrations of eluted dye concentration against albumin concentration. A: main band + tail + trail. B: main band + tail. C: main band only.

EXPERIMENTAL.

Colorimetric determinations of bromphenol blue.

Two instruments were used in the experimental work, an EEL colorimeter with Filter 607, and a Unicam S.P. 400 spectrophotometer set on 595 mμ. Serial dilutions of bromphenol blue gave the calibration curves for optical density shown in Fig. 3. A Hilger Uvispec was not available for routine use in this work, but for comparison a calibration curve for this instrument is also shown.

All the curves in Fig. 3 deviate in varying degrees from the linear relation specified by Beer's Law. Since bromphenol blue shows a sharp absorption peak at 595 mμ, experimental deviation from Beer's Law is to be expected, to an extent depending on the wavelength span of the incident light in the instrument used. Thus the EEL, with only a colour filter, produces marked deviation from Beer's Law, whereas the Uvispec, with a very fine slit width in the monochromator, gives a calibration which is virtually linear in the 0-1 optical density range.

The simpler type of instrument is quite satisfactory for routine use if the colorimetric procedure is carefully standardized and a calibration curve determined for converting optical density to concentration of dye. (It should be noted, in passing, that with marked deviation from Beer's Law the procedure

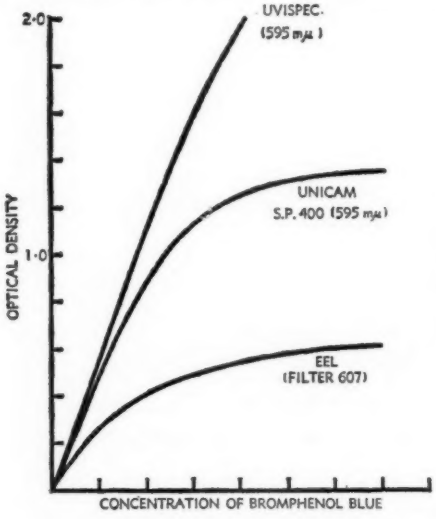

Fig. 3. Calibrations of optical density against concentration of bromphenol blue (arbitrary units), for three instruments as indicated.

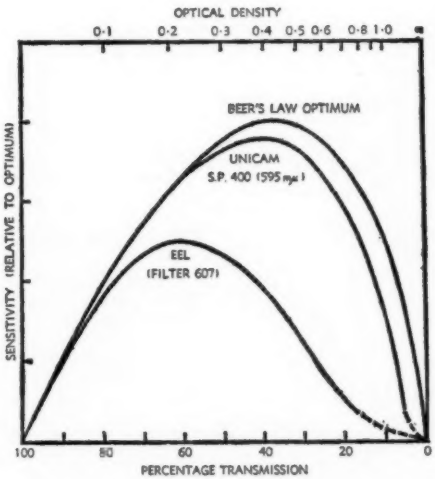

Fig. 4. Sensitivity curves for bromphenol blue determinations on two instruments as indicated, scaled in relation to the optimum sensitivity curve (appropiate when Beer's Law is obeyed) for each instrument.

of setting zero optical density on a coloured blank will not give strictly valid correction. The blank correction should be determined independently.)

Sensitivity. It is well known that the middle range of the spectrophotometric scale gives the greatest accuracy with solutions obeying Beer's Law. A typical sensitivity (reciprocal of relative standard error) curve is shown in Fig. 4, the optimum sensitivity being in the 0·4-0·5 range of optical density

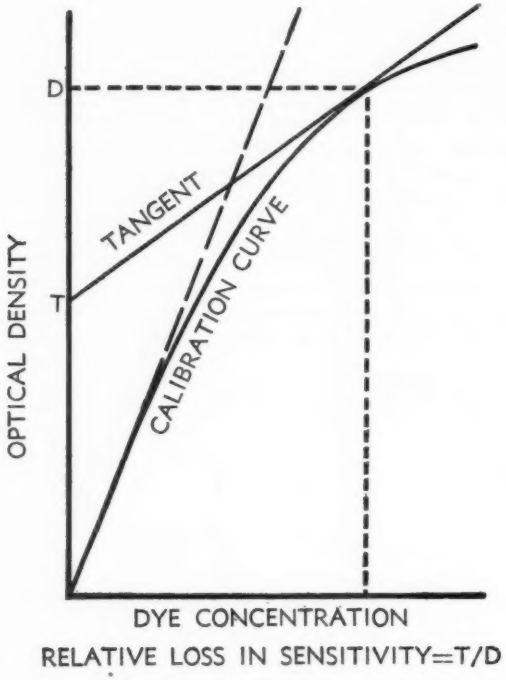

Fig. 5. Graphical determination of the relative loss in sensitivity due to deviation from Beer's Law, from the appropriate calibration curve of optical density. The curves of reduced sensitivity in Fig. 4 were derived by this method.

Departure from Beer's Law induces a loss of sensitivity which may be determined, for each point of the scale, from the appropriate calibration curve by the construction shown in Fig. 5. Two curves of reduced sensitivity are shown in Fig. 4. With marked deviation from Beer's Law, as with the EEL colorimeter, the most sensitive range is displaced towards the zero end of the optical density scale.

Factors affecting the washing process.

For the experiments in this and the following sections, test papers measuring 3 x 4 cm. were prepared by pipetting out 20 μl. amounts of the standard albumin and globulin solutions to form spots of uniform size on the papers. Except in respect to the factor under investigation, or where otherwise specified, processing of the papers (and of similar sized blanks) was carried out as described in the METHODS section above.

Time of washing. A number of test papers for both 2 p.c. and 3 p.c. albumin, together with blanks, were fixed, stained, and then washed together in a solution of 0·5 p.c. (v/v) acetic acid in distilled water, for periods of time varying up to 3 hours. The washing solution was changed at 10-minute intervals. Fig. 6(a) shows the behaviour of the dye during the washing process. The amount of dye held by the test papers decreases steadily during the first hour of washing, after which a stable level is reached and maintained for approximately two hours. The dye held by the blank papers reaches a minimum level (equivalent to 0·04 p.c. albumin over the 12 cm.^{2}) within about 40 minutes.

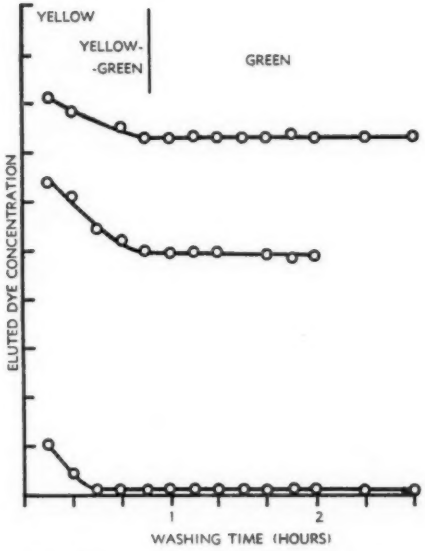

Fig. 6(a). The effect of washing time on protein-bound dye. Washing solution: 0·5 p.c. acetic acid in distilled water. Upper curve: 3 p.c. albumin test papers. Middle curve: 2 p.c. albumin test papers. Lower curve: blank readings. Changes in colour of protein-bound dye are indicated.

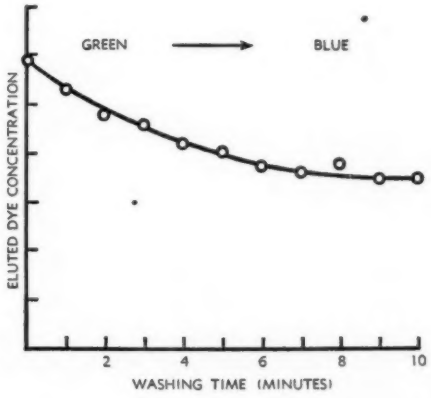

Fig. 6(b). The effect of washing in methanol (see text). Colour changes are indicated.

It was found in this and other experiments that the colour of the dye bound to the protein acts as a reliable indicator for the washing process. The steady decrease in dye level over the first hour is associated with a change in colour from yellow to green. Over the next hour or so, when the dye level is stable, the colour remains a uniform green. After very prolonged washing a gradual change from green to blue may occur, and indicates that further dissociation of dye from the protein is taking place.

Temperatures of the staining and washing solutions. Using 2 p.c. albumin test papers, 16 temperature combinations were tested, covering all combinations of 20, 30, 40, 50° C. for the two solutions. There was no significant effect of either factor on final dye level. However, both factors affected the rate of washing to the stable level, washing being slower at higher staining temperatures but, conversely, faster at higher washing temperatures. In consequence of these opposite effects, very little effect on rate of washing was observed when staining and washing were done at room temperature.

Acidity of the washing solution. Increasing the acetic acid content from 0·5 p.c. to 5·0 p.c. did not appear to affect the final dye level appreciably, but did influence the rate of washing. Washing in distilled water alone gave lower and less stable dye levels.

Methanol washing. Fig. 6(b) shows the behaviour of protein-bound dye when subjected to methanol washing, after a preliminary wash of 20 minutes in the 0·5 p.c. acetic acid solution. Dye is lost rapidly from the protein, and the colour changes from green to blue. Methanol washing was used originally in this laboratory by Paton, Robertson and Wellby (1954), but was found to be difficult to control, being sensitive to slight fluctuations in the pH of the methanol.

Factors affecting the staining process.

Concentration of dye solution. Two sets of albumin test papers were stained in 1 p.c. and 0·1 p.c. solutions of bromphenol blue in 1 p.c. (v/v) ethanolic acetic acid. The concentrations of albumin ranged from 1 p.c. to 6 p.c., which corresponded to a range in protein densities from 0·13 to 0·80 mg./cm.^{2} Fig. 7(a) shows clearly the limiting effect of dye concentration. Under-staining occurred at much lower protein densities with the 0·1 p.c. than with the 1 p.c. dye solution. The results of calibration experiments using the 1 p.c. dye solution (with albumin standards subjected to electrophoresis) showed that, as illustrated in Fig. 2(b), the dye-protein relationship was linear in the calibration range, which corresponded to an estimated maximum density of 0·15 mg./cm.^{2}

In a further experiment, using 1 p.c. γ-globulin and 1 p.c. albumin test papers, dye uptake was examined for several staining solutions in which the concentration of bromphenol blue ranged from 0·02 p.c. to 1 p.c. The protein in this instance was applied to wet papers to form more diffuse spots, giving average densities of approximately 0·03 and 0·06 mg./cm.^{2} respectively on the 1 p.c. and 2 p.c. test papers. The degree of staining (as a percentage of

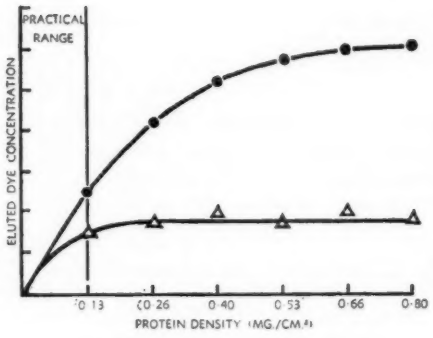

Fig. 7(a). The limiting effect of dye concentration on dye uptake, in relation to protein density. Upper curve: 1 p.c. dye solution. Lower curve: 0·1 p.c. dye solution.

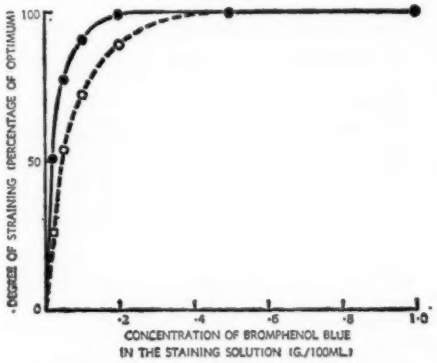

Fig. 7(b). Degree of staining (as percentage of optimum) in relation to concentration of the dye solution. Continuous curve: 1 p.c. γ-globulin test papers. Broken curve: 2 p.c. albumin test papers.

optimum) for both protein densities, in relation to concentration of the dye solution, is shown in Fig. 7(b), which exhibits further the interdependence of dye concentration and protein density in determining the degree of staining; the minimum dye concentration for optimal staining varies with protein density.

Staining time. The effect of varying staining times was examined with both 1 p.c. and 0·1 p.c. dye solutions. In the case of the 1 p.c. solution optimal staining was achieved within 10 minutes for the practical range of protein densities, and very much more rapidly for the lower protein densities, 1-2 minutes sufficing for 1 p.c. albumin test papers (with diffused spot). In the case of the 0·1 p.c. solution, however, dye uptake for higher protein densities was still suboptimal with up to 1 hour of staining. The effect of prolonged staining with the 0·1 p.c. dye solution was not examined.

Influence of the buffer solution. It was found that if the electrophoresis papers were stained without the preliminary washing to remove the sodium barbiturate, the uptake of dye by the proteins was depressed by as much as 20 p.c. This effect persisted when the concentration of acetic acid in the dye solution was increased from 1 p.c. to 5 p.c. Furthermore, failure to remove the sodium barbiturate caused a progressive reduction in staining power with repeated use of the dye solution. It was found expedient, therefore, to fix the proteins first in a mercuric chloride bath and then wash in distilled water prior to staining.

Drying after electrophoresis. Albumin test papers were dried under various conditions before immersion in the mercuric chloride bath: in a forced-draught type oven for 20 minutes and for 30 minutes at temperatures ranging from 35° C. to 100° C.; for 60 minutes at 100° C.; and at room temperature (with fan only) till thoroughly dry. Virtually identical levels of dye uptake were obtained

over this range of conditions. Lower values may occur, of course, at the extremes of accidental scorching or incomplete drying.

Reproducibility.

The results presented in Fig. 2(a) and 2(b) demonstrate the linearity of the dye-protein relationship over the calibration range. Subsequent routine use of the method has provided further confirmation of the linear property and of the overall reliability of the method.

TABLE 1.

Estimated standard deviations of determinations in the same run and in different runs, derived from triplicate determinations of one serum on five consecutive days.

Protein fraction	Mean Estimates g./100ml.	Standard deviation of estimations:			
		Same run		Different runs	
		S.D.	p.c. error	S.D.	p.c. error
α_1	0·05	·015	30 p.c.	·029	60 p.c.
α_2	0·49	·035	7 p.c.	·057	12 p.c.
β	0·86	·034	4 p.c.	·045	5 p.c.
γ	1·78	·033	2 p.c.	·068	4 p.c.
Total globulin	3·18	·060	2 p.c.	·088	3 p.c.
Albumin	4·37	·043	1 p.c.	·076	2 p.c.
Total Protein	7·55	·069	1 p.c.	·069	1 p.c.

Table 1 presents estimated Standard Deviations and the corresponding relative errors for the various protein determinations. These estimates were derived from a series of triplicate determinations on one serum, repeated on five consecutive days using the one staining solution. In the authors' experience the agreement between determinations was perhaps somewhat better on this than on other occasions. Greater day to day variation, in particular, has been experienced. The figures presented in Table 1, nevertheless, give a reasonable idea of the precision attainable with the method, particularly in regard to total protein (S.D. 1 p.c.), determinations of which are virtually unaffected by day to day fluctuations in the degree of resolution of the fractions or of albumin trailing, or by variations in cutting between fractions.

The high degree of relative variability in the α_1-globulin determinations is attributable mainly to the small amount of this fraction present but also, to some extent, to the variability contributed by the correction for albumin tail. It would perhaps be preferable, on this account, to use a single, well-determined correction for albumin tail (as a function of the serum albumin concentration) rather than the more variable corrections determined individually for each run. The same remark does not apply, however, to the correction for albumin trail, which is quite accurately determined in each run.

DISCUSSION.

The most important requirement for accurate estimation of proteins by staining methods is that the uptake of dye per unit of protein should be independent of the density of protein on the paper, for if this condition did not pertain, any factor affecting the density of protein on the paper would affect the estimation. The estimations would become dependent on the amount of serum applied, for instance, and on the specific conditions under which electrophoresis is carried out.

If optimal staining conditions are achieved a linear dye-protein relationship can be obtained for a wide range of protein densities, as has been shown with the staining procedure described in this paper. It is perhaps not so widely recognized that three main factors, *protein density, dye concentration* and *staining time,* are all interdependent in their effects on the degree of staining. To achieve optimal staining it is necessary that both dye concentration and staining time should exceed certain lower limits which are to some extent inversely related to each other and which in combination are both dependent on the maximum protein density in the range for which optimal staining conditions are required. With the staining procedure described in this paper it was found that 10 minutes staining in a 1 p.c. bromphenol blue solution gave optimal staining over the calibration range (up to an estimated 0·15 mg./cm.^{2}). Though the lower limits of dye concentration and staining time are to some extent inversely related, it is possible that with very weak dye solutions optimal staining at higher protein densities may not be achieved even with very protracted staining times; no experimental evidence has been obtained on this point, however, in the present investigation.

A number of authors have used what appear to be sub-optimal staining procedures. Jencks, Jetton and Durrum (1955) standardized on 16 hours' staining in a 0·01 p.c. bromphenol blue solution, though they showed that dye uptake was still increasing after 30 to 40 hours in the staining bath; and Henry, Golub and Sobel (1957), using the staining procedure of Jencks *et al.*, reported non-linearity in dye uptake at higher protein densities. Franglen and Martin (1954), with a staining time of 10 minutes in a 0·2 p.c. solution of bromcresol green, reported gross understaining at higher protein densities. Their actual results (as shown in Fig. 4 of their paper) seem to show, rather inexplicably, that the amount of dye bound to a given area of paper is actually less when there is more protein present. Hardwicke (1954), using a 0·1 p.c. bromphenol blue solution and a staining time of 30 minutes, also found that understaining occurred with higher protein densities (exceeding 0·04-0·05 mg./cm.^{2}), which he attributed to the formation of an impermeable coagulum, and accordingly adopted the expedient of diluting his protein solutions. In this and the other cases cited above, it seems evident that understaining at higher protein densities is largely due to the limiting effect of too low a concentration of dye in

relation to the staining times employed, and in the case of Franglen and Martin this aspect largely vitiates the basis of their attack on quantitative electrophoretic methods.

Many authors have stressed in various degrees the need for rigid control of the staining process to obtain reproducible results. However, the need for such control is symptomatic of sub-optimal conditions. While some factors may affect a process over their entire (practical) range, there exists, for most factors that can affect the process, a mutual set of ranges within which these factors may be varied without influencing the process; the conditions specified by these ranges may appropriately be termed *optimal*, and the degree of control required is governed by the extent of the ranges.

The present investigations, though incomplete in many respects, indicate that the factors, *drying time and temperature, dye concentration and temperature, staining time, washing time and temperature,* and in a more limited sense, *acidity of washing solution,* are all non-critical in the sense described above, requiring only a loose control to ensure that experimental conditions are in the optimal range. These conclusions relate, of course, to the staining procedures as described, and in particular to the kind of staining solution employed. Limited investigations were made of other dye solutions but are not of sufficient extent to report here.

Provided that a good staining method is employed, the influence of the staining process on the accuracy of the determinations is of much less consequence than the factors which affect the electrophoretic process itself, causing variations in the degree of movement and resolution of the various protein fractions and in the amount of albumin trail, for which the globulin estimations need to be adjusted. On account of day to day fluctuations in distance of migration and in the amount of albumin trail, it was found expedient to calibrate with albumin standards subjected to electrophoresis in each run. However, less frequent calibrations of this kind would probably be adequate with more rigid control of the electrophoretic process, as simple test spot calibrations suffice to check on the staining process.

Acknowledgments. The authors are indebted to Dr. W. Roman for his interest in this work, to Mrs. Joan Paton, Dr. R. J. Swaby and Dr. E. Hippsley for helpful discussions, to Mr. E. Liebig for technical assistance and to Mr. Murray Childs who prepared the diagrams. Grateful acknowledgment is made to the Lister Institute for gifts of purified human serum albumin and globulin.

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EQUILIBRIUM SEDIMENTATION OF INFLUENZA VIRUS IN CAESIUM CHLORIDE DENSITY GRADIENTS

by R. D. BARRY

(From the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra).

(*Accepted for publication 21st July, 1960.*)

SUMMARY.

Preparations of standard and incomplete influenza A virus were subjected to equilibrium sedimentation in density gradients of caesium chloride solutions. Concentrated solutions of caesium chloride rapidly destroyed the infectivity of influenza virus.

For standard virus preparations, the distribution of haemagglutinin in these gradients indicates that they consist largely of particles of similar density with a small class of particles of lower density. The size of this class was different in the two strains tested. The distribution of electron-microscopically visible particles obtained for one strain corresponded to the distribution of haemagglutinin. There was no evidence that a separate class of inactive particles exists in standard preparations.

The viral enzyme is uniformly distributed in the virus population.

Incomplete virus preparations lack the homogeneity of standard virus preparations. As incompleteness increases, an array of particle types of variably lower density appears.

INTRODUCTION.

Influenza virus preparations always contain more morphologically characteristic particles, as determined by electron microscopic counts, than can be detected by conventional infectivity titration (Donald and Isaacs, 1954). This discrepancy may result from shortcomings of the assay method or, alternatively, the bulk of the recognizable virus present may be incapable of initiating infection. If classes of particles exist which differ in biological activity, it is possible that such classes also differ in particle density. The technique of equilibrium sedimentation in caesium chloride density gradients (Meselson, Stahl and Vinograd, 1957) provides a means of examining the density homogeneity of macromolecules. In this study, the distributions for standard preparations of influenza A strains MEL and PR8, as well as for several preparations of incomplete virus, have been determined in such density gradients. Concentrated caesium chloride solutions rapidly inactivate influenza virus infectivity, so the presence of virus was detected by haemagglutination. For standard virus, the distribution of virus enzyme and electron-microscopically visible particles was also determined.

Materials and Methods.

Strains of virus. The following strains of influenza virus were used:
PR8 (Influenza A virus strain, isolated by Francis, 1934).
MEL (Influenza A virus strain, isolated by Burnet, 1935).

Preparation of virus. Eleven-day-old eggs were inoculated allantoically with 10^2-$10^4 ID_{50}$ of virus. After incubation at 36° C. for 24 to 30 hours, they were chilled, harvested and the fluids pooled. This provided preparations of active virus with high ID_{50}/HA ratios, which will subsequently be referred to as standard virus.

In centrifugation experiments, either unconcentrated allantoic fluid preparations of virus or concentrates prepared by sedimentation in the ultracentrifuge and resuspension were employed. A concentration of one hundredfold was achieved in the latter case. Examination of concentrated preparations in the electron microscope revealed, however, that extensive aggregation occurs. Treatment of these preparations for one minute with a Mullard 50 watt ultrasonic drill, produces a state of dispersion indistinguishable from that found in untreated allantoic fluid preparations.

To obtain incomplete virus, serial passage of undiluted virus was initiated, as described by von Magnus (1951), commencing with standard virus of high titre. Usually, 0·2 ml. of undiluted allantoic fluid was used as inoculum, and eggs were incubated for 24 hours before harvesting.

Infectivity titrations. Infectivity titrations were carried out by the method of Fazekas de St. Groth and White (1958), in pieces of surviving allantois-on-shell, using 3·16 fold dilution steps of test material and ten replicates per dilution step.

Haemagglutination titrations. Haemagglutination (HA) titrations were carried out in perspex trays with a Takatsy loop as described by Fazekas de St. Groth and Graham (1955), except that 0·25 ml. volumes of 1 p.c. fowl red cell suspensions were added by means of an automatic pipetting machine.

Titration of virus enzyme. Tests for enzyme activity were determined using ovomucin as substrate. The ovomucin was prepared from egg white and titrated for inhibitory activity as described by Gottschalk and Lind (1949). The tests for enzyme activity followed that described by Stone (1948), in which five inhibitory units of ovomucin were incubated with serial two-fold dilutions of virus at 37° C. for one hour, followed by 30 minutes' heating at 65° C. to destroy further enzyme activity. Residual inhibitory activity was then determined by addition of five agglutinating doses of heat-inactivated LEE virus, followed 30 minutes later by 0·25 ml. of a 1 p.c. suspension of "sensitive" fowl red cells (Anderson, 1948).

Electron microscopy. In preparing samples for electron microscopy, the agar filtration method of Kellenberger and Arber (1957) was adopted. Collodion films formed over agar surfaces serve as a filter through which liquids and salts will rapidly diffuse. Mixtures of virus and standardized polystyrene latex are spread over such a surface, and filtration occurs quickly. The preparation is fixed by inverting the plate over a small amount of osmic acid for fifteen minutes. After fixation, that portion of the collodion covered by the dried film is cut into small pieces, about 5 $mm.^2$ in size, and floated off the agar onto copper electron microscope grids. Routinely, 0·2 ml. volumes of the mixture were spread, the latex particles being 365 mμ in diameter, and used at a concentration of $2{\cdot}25 \times 10^{10}$ particles per ml. Samples were shadowed with uranium and photographed in an RCA EMU-3B electron microscope.

The numbers of each type of particle and their ratios obtained from single micrographs vary considerably because of the variation in thickness of the spread film. However, Kellenberger and Arber (1957) have shown that five counts made from different fields of the specimen are sufficient to obtain precise particle counts.

Density gradient centrifugation. A Spinco model L ultra-centrifuge was used with the swinging bucket rotor SW 39L. Preliminary experiments indicated that an initial density of 1·24 gm./ml. for the virus-caesium chloride mixture, followed by centrifugation at 33,000 r.p.m. for a 40-hour period, resulted in a stable density gradient, with the virus located near the middle of the centrifuge tube. The difference in density between the top and the bottom of the tube under these conditions is about 0·1 gm./ml. Centrifugation was carried out in lusteroid tubes, of 5 ml. capacity, and at the completion of a run, samples were collected by piercing the bottom of the tube with a pin and collecting the contents as single drops.

RESULTS.

The distribution of haemagglutinin. Banding of virus in a density gradient was carried out as described. Each drop sample obtained from the tube was collected into 1 ml. volumes of distilled water. Tests for haemagglutinin were carried out on each. For graphical comparison of the distribution of haemagglutinin in various preparations, all curves are plotted as being of equal area.

(a) *Standard virus.* Representative distributions of haemagglutinin for the A strains MEL and PR8 at equilibrium are shown in Figs. 1 and 2.

Meselson, Stahl and Vinograd (1957) have shown that the concentration distribution at equilibrium of a single macromolecular species is Gaussian, whereas the distribution becomes skewed in the presence of material heterogeneous with respect to effective density. Standard MEL virus is characterized by a slightly skewed, unimodal band, but about 90 p.c. of the total haemagglutinin is concentrated in a narrow band, while the remainder presents a heterogeneous array of lower densities. With standard PR8 virus, the distribution is

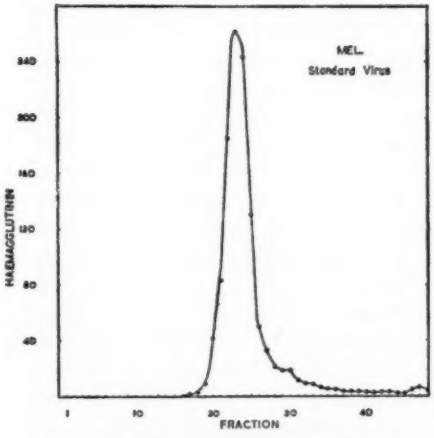

Fig. 1. The equilibrium distribution of haemagglutinin for a standard MEL preparation in a CsCl density gradient. The initial density of the virus-caesium chloride mixture was 1·24 gm./ml.

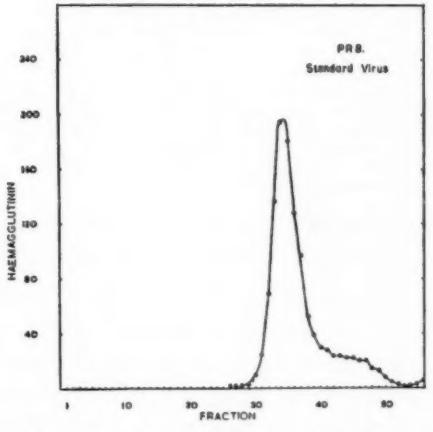

Fig. 2. The equilibrium distribution of haemagglutinin for a standard PR8 preparation in a CsCl density gradient.

considerably more skewed, with approximately 20 p.c. of the virus material constituting the tail region. Again, however, the bulk of the haemagglutinating activity is in a narrow band. It is possible that this distribution may be bimodal, characterized by a high peak representing a relatively homogeneous virus fraction, overlapped by a diffuse distribution of particles heterogeneous in respect to density, with the differences in effective density being insufficient to result in the formation of discrete bands. On the other hand, it was considered possible that at least part of the skewness of these distributions may have been due to the method of sampling. Consequently, sample No. 35 from the homogeneous peak of the PR8 run was rebanded and again examined for haemagglutinin distribution. Fig. 3 shows the distribution obtained. Clearly, this curve indicates that an almost normal distribution can be expected from the banding of a homogeneous species, and that very little mixing occurs during sampling.

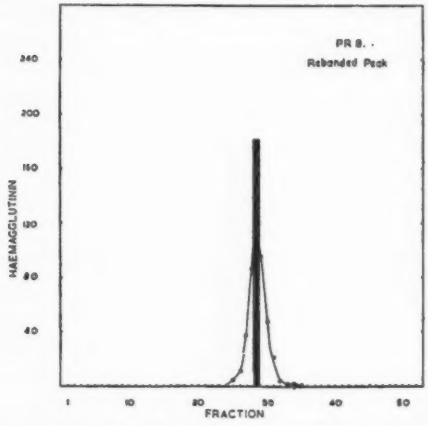

Fig. 3. The rebanding of material showing density homogeneity. The equilibrium distribution obtained for fraction No. 35 of the standard PR8 preparation shown in Fig. 2. The black band indicates the original area occupied by this sample.

Hence the haemagglutinin distributions for normal virus preparations show that while the bulk of the virus haemagglutinin is fairly homogeneous with respect to density, there is a variable amount present depending on the strain, which is less dense than the rest.

(b) *Incomplete virus.* Stocks of first and second passage incomplete PR8 virus were prepared, and subjected to density gradient centrifugation. The results obtained for first passage virus are shown in Fig. 4. Once again a unimodal skewed distribution occurs, but the peak of haemagglutinin activity is not nearly so prominent as in standard virus preparations and is considerably wider, suggesting that the bulk of this type of virus is more heterogeneous with respect to density than is standard virus. The diffuse class of particles of lower density found with standard virus is present once more. With further passage, the heterogeneity of density increases, as shown by second passage incomplete virus in Fig. 5. In this case, no uniform fraction can be distinguished, and the resultant curve can best be described as representing a broad spectrum of density types, with a general shift towards lower density. A very light fraction is also present which floats on the top of the gradient. Consequently, the lowering of infectivity and loss of morphological uniformity which have been

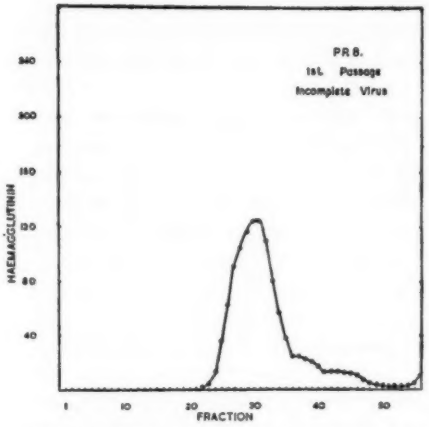

Fig. 4. The equilibrium distribution of haemagglutinin for a first passage incomplete virus preparation of PR8.

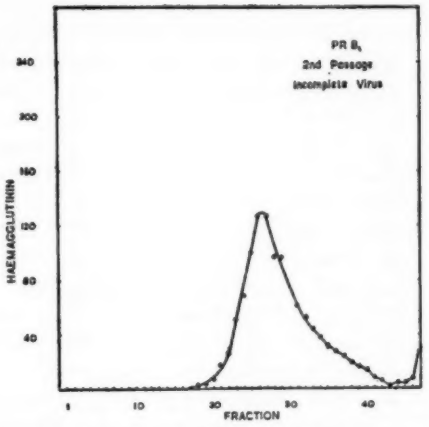

Fig. 5. The equilibrium distribution of haemagglutinin for a second passage incomplete virus preparation of PR8.

observed to occur on undiluted passage of standard virus preparations are associated with a marked increase in heterogeneity of particle density. Fig. 6 is a composite of standard, first and second passage incomplete virus plotted to demonstrate these alterations in density heterogeneity on passage. In the

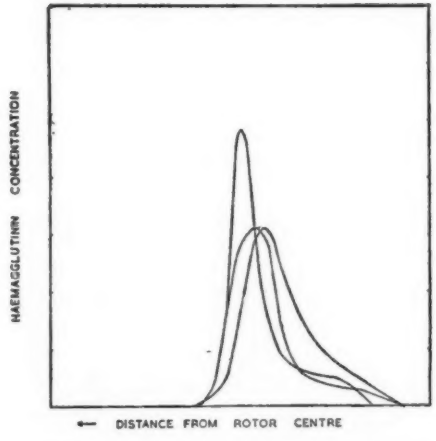

Fig. 6. The relative equilibrium distributions of standard virus and first and second passage incomplete virus preparations of PR8.

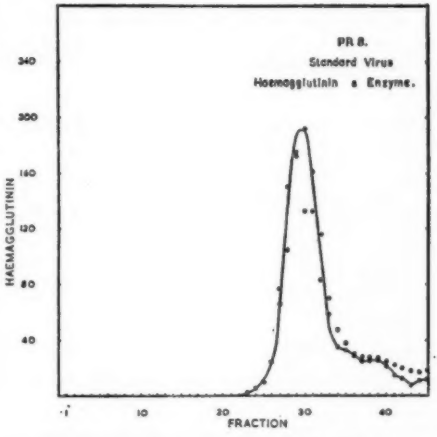

Fig. 7. The equilibrium distribution of virus enzyme and haemagglutinin for a standard PR8 preparation. Closed circles represent the values obtained for enzyme; open circles indicate the values obtained for haemagglutinin.

originals, the position of each band in the tube varies slightly, owing no doubt to small, unavoidable differences in the initial densities of the mixtures. In this Figure, they have been adjusted by assuming that the first detectable appearance of haemagglutinin occurs at similar densities.

The distribution of enzyme. After density gradient centrifugation of standard virus preparations, each sample was tested for the presence of virus enzyme, and the distribution obtained was compared to that found for haemagglutinin. Fig. 7 is a plot of a typical experiment in which the actual enzyme values have been adjusted so that a curve of equal area to that for haemagglutinin could be obtained. The distribution of enzyme appears to be almost exactly superimposed on that for haemagglutinin. Individual values for enzyme sometimes differ quite markedly from their corresponding haemagglutinin values, but this is probably a reflection of the inherent variability of the test employed to detect enzyme.

The distribution of electron-microscopically visible particles. The only distribution of particles examined was obtained from the fractions of a standard MEL preparation after density gradient centrifugation (Fig. 2). This was an unfortunate choice in searching for possible particle heterogeneity, as the haemagglutinin distribution for strain MEL is much more homogeneous than for PR8. Particle counts were undertaken on samples 19, 21, 23, 25, 27, 29, 31 and 47 of the MEL distribution shown in Fig. 4. Other samples were taken from the tail region, but there were too few particles present for counts to be made. Table 1 lists the number of visible particles per ml. found for this strain before centrifugation and for the various fractions examined, as well as the ratios of particles to haemagglutinin. Columns showing the actual number of virus and latex particles counted are included in this Table.

TABLE 1.

The relation of the distribution of total particles (photographed with the electron microscope) to the distribution of haemagglutinin after density gradient centrifugation.

MEL preparation	V[a]	L[b]	P/ml($\log_{10}$)[c]	HA/ml($\log_{10}$)[d]	P/HA($\log_{10}$)[e]
Stock	138	358	9·94	3·14	6·80
No. 19	43	256	9·60	2·86	6·74
No. 21	673	492	10·48	3·81	6·67
No. 23	160[f]	203	11·26	4·31	6·95
No. 25	115[f]	240	11·04	4·01	7·03
No. 27	381	241	10·60	3·41	7·19
No. 29	269	440	10·15	3·16	6·99
No. 31	52	236	9·69	2·96	6·73
No. 47	273	404	10·18	2·68	7·50

(a) Virus particle count obtained from five fields.
(b) Latex particle count obtained from five fields.
(c) Virus particle count per ml.
(d) Haemagglutinin titre.
(e) Number of particles representing one agglutinating unit.
(f) Virus preparation diluted tenfold before mixing with latex.

The ratio of particles to haemagglutinin varies over a twofold range throughout the distribution, with the exception of sample 47, and when the particle distribution is plotted against the haemagglutinin distribution, as shown in Fig. 8, the correspondence between the two is very close. However, at the extreme right-hand end of the distribution, the number of particles required for haemagglutination appears to be much higher than previously. Electron-micrographs of virus located in this portion show that there is much greater variation in the size of particles than in the peak samples. The presence of large numbers of particles rather smaller than the virus particle was responsible for the high particle to haemagglutinin ratio. It is very likely that many of these particles do not represent virus, but particulate matter of lower density which floats at the top of the density gradient. Consequently, in plotting the relative distributions of particles to haemagglutinin shown in Fig. 8, sample 47 was not considered. The overall impression gained from particle counts is that the distribution of visible particles is identical with the distribution of biological activity, i.e. there is no evidence for the existence of a class of biologically inert particles, which have a different density distribution from the biologically active particles.

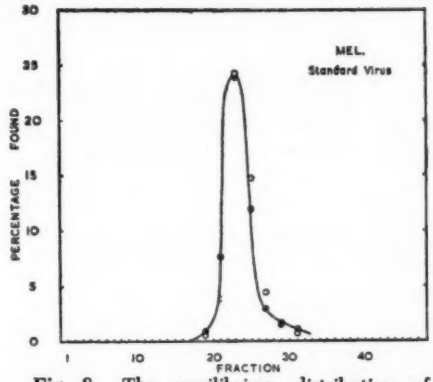

Fig. 8. The equilibrium distribution of total particles visible in electron micrographs and haemagglutinin for a standard MEL preparation. The open circles represent the distribution of visible particles, while the closed circles represent the distribution of haemagglutinin.

Inactivation of infectivity by caesium chloride. It was hoped that after virus was banded in caesium chloride (CsCl) density gradients, it would be possible to determine the distribution of infectivity, particularly for incomplete virus preparations. However, it was found that storage of virus in the high concentrations of CsCl needed to achieve a density of 1·24 gm./ml., resulted in a marked loss of infectivity. That inactivation was not due to high ionic strength alone was borne out by the fact that storage of virus in similar concentrations of sodium chloride did not result in any marked loss of infectivity. Infectivity also remains high if virus is kept in physiologically "normal" (0·147M) CsCl solutions. However, all attempts to associate this inactivation with a trace impurity, or to counteract the effect empirically, failed.

That the inactivation effect was due to the caesium ion was shown by the partition of CsCl on a cation-exchange resin column. Caesium chloride was added to an acidified Dowex-50 resin column, and the chloride of the salt passed through the column in the form of hydrogen chloride. This was converted to

sodium chloride with sodium hydroxide, and evaporated to dryness. This fraction contained the original chloride ions of the caesium salt. Caesium was then removed from the column by addition of 50 p.c. hydrochloric acid. This fraction was also dried. The two dried fractions were now made up into solutions of equivalent strength to that of stock CsCl solution (6M), and tested for their capacity to inactivate virus. The effect of the various fractions in inactivating infectivity is shown in Table 2 where X represents in each case the ions obtained by partition. The mixtures contained PR8 infected allantoic fluid, and were stored overnight in the refrigerator before testing for infectivity. The amounts shown in each column represent the volume in ml. of each constituent.

TABLE 2.

Identification of the agent responsible for inactivation of influenza virus infectivity by CsCl.

Sample	Constituents						Infectivity titre $\log_{10}$
	Virus	Distilled water	CsCl 6M	NaCl 6M	XCl[a] 6M	NaX[b] 6M	
Control	0·1	0·2	—	—	—	—	7·2
CsCl	0·1	0·1	0·1	—	—	—	4·6
NaCl	0·1	0·1	—	0·1	—	—	6·8
XCl	0·1	0·1	—	—	0·1	—	4·9
NaX	0·1	0·1	—	—	—	0·1	7·0

(a) X=Cs derived from column (see text).
(b) X=Cl derived from column (see text).

Clearly, extensive inactivation occurs only with those samples which contain a high concentration of cations.

DISCUSSION.

Influenza virus has a density of about 1·25 gm./ml. in caesium chloride solutions, a value which is higher than that of 1·19 gm./ml. found in sucrose solutions (Lauffer and Taylor, 1953). This is probably due to the penetration of the mantle of hydration of the virus particle by caesium ions.

Although both tobacco mosaic virus and bacteriophage have been banded by this method without loss of infectivity (Meselson, Stahl and Vinograd, 1957), and concentrated solutions of caesium chloride do not affect the infectivity of poliovirus or vaccinia, it has been impossible to prevent the inactivation of influenza virus. The reason for this inactivation is not clear.

Standard virus preparations consist mainly of particles of uniform density. There is a small percentage, however, which is less dense than the remainder. This is much more prominent with PR8 than MEL, and is probably identical

with the lighter fraction of virus found with PR8 preparations on direct sedimentation in the analytical ultracentrifuge (Friedewald and Pickels, 1944; Lauffer and Stanley, 1944; Hanig and Bernkopf, 1950; Gard *et al.*, 1952; Paucker *et al.*, 1959). Similarly, the heterogeneity encountered in incomplete virus preparations parallels that found on direct sedimentation (Gard *et al.*, 1952).

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CHANGES IN POLARISATION RESISTANCE DURING THE REPOLARISATION PHASE OF THE RABBIT VENTRICULAR ACTION POTENTIAL

by E. A. JOHNSON AND J. TILLE

(From the Department of Pharmacology, University of Sydney, New South Wales),

(*Accepted for publication 21st October, 1960.*)

SUMMARY.

The magnitude and time course of changes in polarisation resistance during the repolarisation phase of action potentials recorded from rabbit ventricular muscle have been measured with intracellular double-barrelled microelectrodes.

Action potentials from 74 fibres in 7 preparations have been examined. In all fibres, at some time during the repolarisation phase, the polarisation resistance was greater than the resting polarisation resistance. The mean maximum increase above the resting value was 1·63 (± 0·15 S.D.) times.

The polarisation resistance remained constant even with large negative voltage displacements (60 mV). No evidence was found of regenerative repolarisation.

INTRODUCTION.

One of the important pieces of information required to formulate a hypothesis about the prolonged repolarisation phase of the cardiac action potential, is the time course and magnitude of associated changes in membrane resistivity. These are reflected as changes in polarisation resistance, i.e. resistance to the flow of current from an intra-cellular microelectrode to a distant point outside the fibre. This paper describes our measurements of the magnitude and time course of such changes in the fibres of the isolated right ventricle of the rabbit.

METHODS.

Details of the preparation, apparatus and experimental technique have been described (Johnson and Tille, 1961). The tissue was stimulated at a rate of 3·4 sec.$^{-1}$

The method of determining the polarisation resistance is based on that described by Frank and Fuortes (1956) for determining the resting polarisation resistance of motoneurones and is the one which we have previously used to determine the resting polarisation resistance of rabbit ventricular fibres (Johnson and Tille, 1961).

We have shown in the same paper that the fibres of the rabbit right ventricle can be divided into two groups (P and V fibres). P fibres have a mean resting polarisation resistance of 106 Kohms and exhibit a linear relationship between action potential amplitude and all values of hyperpolarising current. V fibres have a mean resting polarisation resistance of 47 Kohms and their action potential amplitude remains constant with moderate values of

applied hyperpolarising current. We have confined our experiments to P fibres as the high values of current (10^{-6} A) which would be necessary in the case of V fibres would make the experiments much more difficult. Due to the drop in polarisation resistance which occurs during the rising phase and to the fact that the membrane time constant of P fibres is longer than the duration of the rising phase (Johnson and Tille, 1961), the voltage displacement at the crest of the action potential is relatively small and can be neglected without introducing a serious error.

Definitions.

(1) By polarisation resistance we mean the ratio of the change in membrane potential, in volts, produced by a given current (at an infinite time after the start of the current and at zero distance from its site of application) and the current strength in amps.

(2) Action potentials recorded in the absence of polarising current are called normal action potentials.

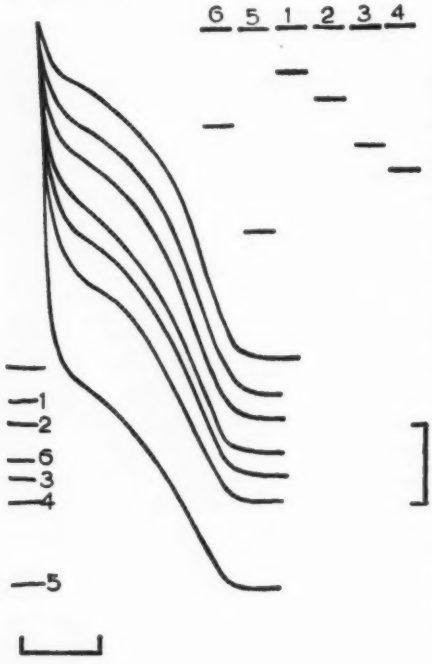

Fig. 1. A tracing of a normal action potential (uppermost of the group) and of action potentials modified by various values of hyperpolarising current. They are superimposed in such a way that the crests are coincident. The numerals to the left of the action potential traces refer to the corresponding values of current shown by the numbered horizontal bars in the upper right of the Figure. Vertical calibration: 25 mV and 0·25 μA; horizontal calibration: 50 msec.

RESULTS.

Constant rectangular pulses of hyperpolarising current were passed through the cell membrane *via* one barrel of a double-barrelled microelectrode. The pulses were arranged to begin approximately 80 msec. before the upstroke of every eighth action potential and ended at least 40 msec. after the conclusion of the repolarisation phase. The resultant potential changes were recorded by the other barrel of the microelectrode. The maximum value of current used in most fibres was such as to produce potential displacements of up to 80 mV. At least four strengths of hyperpolarising current were used with each action potential. The effect of each value was recorded separately. The separate records, together with a record of a normal action potential, were then traced and superimposed in such a way that the crests (where no significant potential displacement was produced by the current) were coincident (Fig. 1). In such a multiple tracing the vertical displacement between the normal action potential and one occurring during current flow is at any time proportional to the potential difference pro-

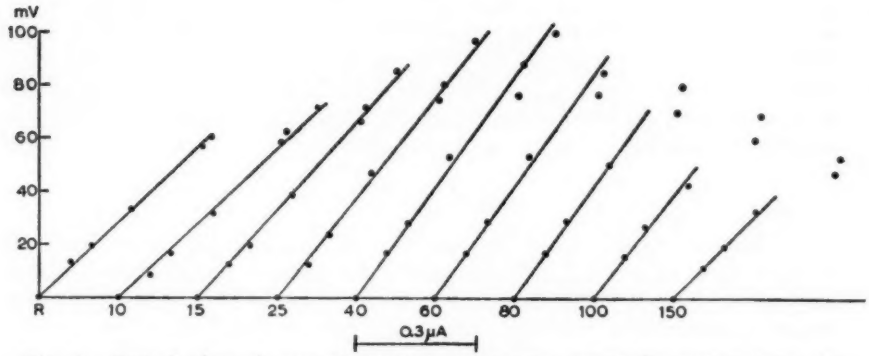

Fig. 2. Typical plots of potential displacement against current for various times (in msecs, given by the numerals on the horizontal axis) after the upstroke of the action potential. R. refers to the plot for the resting membrane just prior to the upstroke.

duced by the current at that time. These displacements were measured at given times after the upstroke of the action potential and were plotted (expressed as potential differences) against the corresponding values of current. An example of a set of such plots is shown in Fig. 2.

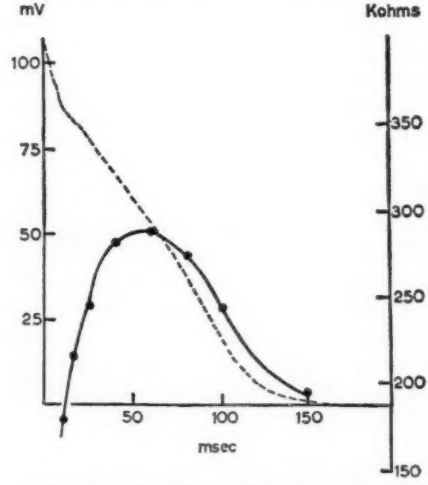

Fig. 3. Polarisation resistance against time shown in time relation to the corresponding action potential. The abscissa meets the right ordinate scale at the value of the resting polarisation resistance.

The current-voltage relationship was substantially linear over the range of currents used. Some non-linearity, frequently but not always, occurred at some time during the repolarisation phase of the action potentials. Fig. 2 illustrates a typical form of non-linearity which is seen to be confined to the later stages of the repolarisation phase and the higher values of current.

The slopes of the current-voltage relationships (expressed as polarisation resistances) were plotted against the corresponding time intervals and an example is shown in Fig. 3. Action potentials from 74 fibres in 7 preparations were examined in this way. In all fibres the drop in polarisation resistance which occurred during the upstroke of the action potential disappeared rapidly so that within 10 msec. the polarisation resistance had increased to its resting value. Thereafter the polarisation resistance continued

to increase, reaching a mean maximum for 74 fibres of 1·63 (± 0·15 S.D.) times the resting value at some time during the repolarisation phase. The mean resting polarisation resistance was 93 (± 44 S.D.) Kohms and the mean maximum polarisation resistance was 144 (± 62 S.D.) Kohms. In the terminal phase of repolarisation the polarisation resistance returned to the value obtained just prior to the upstroke.

DISCUSSION.

Weidmann (1951, 1957) has shown that the polarisation resistance of Purkinje fibres of the kid is greater during the plateau of the action potential than during diastole. Inspection of Fig. 2 of Weidmann (1951) shows that the maximum polarisation resistance during the plateau is 1·4 times greater than the polarisation resistance immediately before the upstroke. This value is close to the value of 1·63 (± 0·15 S.D.) found in the present experiments for the ratio of the maximum polarisation resistance during the plateau and the polarisation resistance just before the upstroke.

Rosenbleuth and del Pozo (1943), Eyster and Gilson (1947) and Cranefield, Eyster and Gilson (1951) using impedance bridge measurements with external electrodes found an increase in membrane resistivity associated with cardiac activity. However, Coraboeuf, Zacouto, Gargouil and Laplaud (1958) reported that in the guinea-pig ventricle the polarisation resistance during the repolarisation phase is approximately half the resting polarisation resistance. Our results and those of Weidmann support the hypothesis that during the repolarisation phase of the cardiac action potential the membrane conductance to potassium ions decreases, returning to its resting value towards the end of the action potential.

Weidmann (1951), in kid Purkinje fibres and Cranefield and Hoffman (1958) in the dog ventricle have reported that hyperpolarising current of sufficient strength applied during the repolarisation phase abolishes the action potential. Our results show that such regenerative repolarisation does not occur in the rabbit ventricular fibres which we have examined. Even with currents which produced potential displacements of 60-80 mV, no discontinuity occurred in the current-voltage relationship. This would suggest that the net membrane ionic conductance is not particularly voltage-dependent during the repolarisation phase of rabbit ventricular action potentials.

Acknowledgments. The authors wish to thank the Life Insurance and Medical Research Fund of Australia and New Zealand for a grant in aid of this work.

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THE FUNCTION OF TOTAL DOSE IN THE PRODUCTION OF CHRONIC LETHAL LIVER DISEASE IN RATS BY PERIODIC INJECTIONS OF THE PYRROLIZIDINE ALKALOID, HELIOTRINE

by L. B. BULL AND A. T. DICK

(From the Division of Animal Health, C.S.I.R.O., Animal Health Research Laboratory, Parkville, Victoria).

(*Accepted for publication 31st August, 1960.*)

SUMMARY.

Groups of young male rats were given 0·1 LD_{50} heliotrine intraperitoneally three times per week for seven weeks. One group was then killed for assessment of liver injury, and other groups were killed and similarly examined at 10, 15, 20, 25 and 30 weeks after the last injection. At each of these time intervals also one group which had been given the first course of injections and one which had not, were started on a course of injections at the same dose and frequency as the first, but injections were continued until each rat died.

The mean total dose required to kill the rats was found to be the same whether it was given in one continuous course of 0·1 LD_{50} injections or broken into two courses with periods of 10-30 weeks between them. With increasing age, however, an increase in susceptibility of the rats to the alkaloid became apparent.

Histopathological examination of the livers revealed that in the absence of further dosage beyond 2·1 LD_{50}, the lesion continued to progress for 20 weeks, but subsequently became modified by an increasing amount of active parenchymal regeneration. The effects of the second course of injections on the lesion already existing was to increase the rate of its development; it became more chronic and further advanced at the time of death in all groups given the broken than in those given the unbroken courses. From these observations it seems that the determination of failure of liver function in the chronic liver disease is to be sought in the biochemical rather than in the morphological changes in the liver.

INTRODUCTION.

The consumption of *Heliotropium europaeum* by sheep leads to their developing a chronic liver disease which is fatal in the majority of the animals (Bull, Dick, Keast and Edgar, 1956). There are usually no untoward results when sheep graze this summer-growing annual for the first season. If they graze it for the second time, eight or nine months later, deaths start to occur in the flock from one to three months after the consumption of the plant has ceased.

The plant contains hepatotoxic pyrrolizidine alkaloids including heliotrine, lasiocarpine, and their N-oxides (Culvenor, 1954; Culvenor, Drummond and Price, 1954; Crowley and Culvenor, 1956). The pure alkaloids have been used for the study of their hepatotoxic action in rats (Bull, Dick and McKenzie, 1958; Bull and Dick, 1959).

In a more recent experiment with rats, we have sought an explanation for the apparent increased susceptibility of sheep to a second grazing on the plant after a "rest" period of eight to nine months. Heliotrine was used as the test substance. The objective of the experiment reported in this paper was to determine the mean chronic lethal dose of heliotrine when given in repeated small doses to rats which had a previous experience of the alkaloid, and to compare this with that obtained in the same way with rats of the same age, but without previous experience. The hepatotoxic action of the alkaloid under the varying conditions of age of the rat and total dosage of the alkaloid was studied by histopathological methods.

MATERIAL AND METHODS.

These are similar to those already described by Bull and Dick (1959). The stock grain-mash diet was used throughout.

Design of the experiment.

A batch of 134 young male rats of a hooded strain, 70-76 days old, was used. The dose of heliotrine given was based on the acute LD_{50}, and was the same, namely, 0·1 LD_{50} (30 mg./kg.), as that used in earlier studies (Bull and Dick, 1959) on the production of the chronic liver disease in the rat. The dose was given by intraperitoneal injection and was adjusted to body-weight changes each week.

The rats were divided into 16 groups which, for the purpose of description, are arranged in three series. One, the (a) series, consisted of 5 groups of 10 rats each, designated group 1(a) to group 5(a). Another, the (b) series, consisted of 6 groups of 5 rats each, designated group 1(b) to group 6(b). Still another set, the (c) series, consisted of 5 groups of 10 rats each, designated group 1(c) to group 5(c).

Both the (a) and (b) series of groups were subjected to a preliminary course of intraperitoneal injections of 0·1 LD_{50} heliotrine 3 times a week for 7 weeks, giving a total dose of 2·1 LD_{50} heliotrine. Rats in the (c) series did not receive this 7 weeks' course of injections, but were used later for comparison with the (a) series which did receive it.

Three days after the last injection to the (a) and (b) series, group 6(b) was killed. Then, at each of the periods 10, 15, 20, 25 and 30 weeks later (i) one of the (b) groups was killed, (ii) one of the (a) groups was started on a second or main course of injections of 0·1 LD_{50} heliotrine 3 times per week, and (iii) one of the (c) groups, which had not been given any injections previously, was started on a similar course. Consequently as the intervals between courses rose from 10-30 weeks there was a progressive increase in age of the rats in groups 1(a) to 5(a) and 1(c) to 5(c) at the time they were started on the final or main course of injections. These injections were continued until the death of the individual rats.

RESULTS.

The lethal effects.

All the rats in the (a) and (b) groups survived the first course of injections in which the total dose received was 2·1 LD_{50} over a period of 7 weeks.

The results of the experiment showing the effect of the previous course, of increased intervals between the first and second courses, and of increased age of the rats on the chronic lethal dose are summarized in Table 1.

Most of the rats in these groups were able to complete their term in the experiment. Of the original 100 rats in the 10 groups shown in Table 1, 6 only have been excluded on the score of doubtful specific death.

The dose required in the second course of injections to cause death in the groups in the (a) series, which had received the earlier course of 2·1 LD_{50} 10 to 30 weeks previously, was always smaller than that required to cause death of the rats in the corresponding groups in the (c) series which had not received the earlier course. The mean lethal doses of the (a) and the (c) series were in the ratio of 1 to 1·64.

However, the mean total lethal dose given in the two courses to the (a) series (5·27 LD_{50}) was not significantly different from that required to kill rats in the (c) series (5·17 LD_{50}) given in one continuous course. Therefore, within the limits of time used in the experiment, death from chronic liver disease produced by heliotrine depended on the total amount (expressed as LD_{50}) of alkaloid received by the rats. This is the more interesting if we take into consideration the findings of our colleague, A. T. Dann (unpublished), that a large proportion of a dose of heliotrine, about 1 LD_{50}, can be accounted for by excretion in the urine within 18 hours.

When the interval between the first and second courses of injection was increased from 10 to 30 weeks, the mean total dose for a lethal result was reduced from 5·4 to 4·4 LD_{50}. In the (c) groups which had not received the first course of injections there was a comparable fall from 5·9 to 4·6 LD_{50} in the period of 20 weeks.

The results obtained with the (c) groups on an unbroken course of injections show that there is a rise in susceptibility to the lethal effect of the alkaloid with increasing age of the rats. Statistical analysis shows that there is a highly significant linear regression of dose on age in days which is expressed as Dose $= -0{\cdot}0099$ Age $+ 7{\cdot}79$. The data show no significant quadratic trend. The coefficient of the correlation of lethal dose with age is $-0{\cdot}488$. It is not possible to determine from the data, however, whether the susceptibility increases steadily with age or whether rats over a certain age are more susceptible than rats under it.

Pathological effects.

The chronic and progressive pathological effects on the liver of the rat of repeated small doses of heliotrine and other alkaloids of *H. europaeum* have already been described (Bull and Dick, 1959) as follows. The histopathological changes are characterized by the increase in size of the nucleus and of the cytoplasm of the hepatocytes (megalocytosis). The megalocytosis is zonal in distribution in the early stages and becomes generalized later when new bile-duct formation and atrophic changes become manifest, with little increase in

TABLE 1.

The effect on the chronic lethal dose of heliotrine of (i) previous course, (ii) increased intervals between first and second course, and (iii) increase in age of rats.

Group	No. rats	Mean age (days)			Dose (x LD_{50})				Interval between finish of 1st and start of 2nd courses	
		At start of		At death	1st course	2nd course	Total*		(weeks)	(days)
		1st course	2nd course				Mean	Range		
1(a)	10	75	194	275	2·1	3·3	5·4	3·9—6·3	10	(73)
1(c)	10	73	192	339	—	5·9	5·9	4·4—7·2	—	
2(a)	9	73	229	313	2·1	3·4	5·5	4·1—6·8	15	(110)
2(c)	10	76	232	363	—	5·3	5·3	2·4—6·9	—	
3(a)	10	72	261	365	2·1	4·0	6·1	4·8—7·5	20	(143)
3(c)	10	73	262	394	—	5·7	5·7	3·8—7·1	—	
4(a)	8	76	300	364	2·1	2·7	4·8	4·0—5·4	25	(178)
4(c)	10	74	298	400	—	4·3	4·3	3·1—5·0	—	
5(a)	9	71	330	386	2·1	2·3	4·4	3·3—5·7	30	(213)
5(c)	8	73	332	439	—	4·6	4·6	3·0—5·1	—	
Total	94									
	Mean of 2-course groups					3·17	5·27			
	Mean of 1-course (control) groups					5·17	5·17			

* Difference required for significance between mean lethal doses of any two groups of rats of different ages in the (c) series is 0·85 at 5 p.c. level, and 1·14 at 1 p.c. level.

fibrous tissue. The maximum megalocytosis, as assessed by the proportion of nuclei enlarged and size of nucleus, or from the number of cells per unit volume of tissue, was found to be largely a function of time. It and the most chronic lesions were produced if the repeated small doses of alkaloid were broken from time to time by short rest periods when alkaloid administration was stopped temporarily.

The present experiment was designed to provide more information on the effects of longer rest periods. The main pathological lesions in the liver produced by the first course of injections in which a total dose of 2·1 LD_{50} heliotrine was given, and the progress of these lesions with time, or with further dosage of the alkaloid, are summarized in a semi-quantitative manner for comparative purposes in Table 2.

Histopathological examination of the (b) series of groups shows that at the end of the first course of injections there is some enlargement of the hepatocytes in the midzone and periphery of the lobule, but the centrilobular cells remain small and appear unaltered. Ten weeks later megalocytosis has become more general, leaving only relatively few unchanged centrilobular cells, and there is some new bile-duct formation. At fifteen weeks the lesion has progressed still further with added hyperplasia of the cholangioles, but a slight degree of parenchymal regeneration, in the form of a few circumscribed miliary areas, has appeared.

At twenty weeks, there is further cholangiole hyperplasia, the small centrilobular cells are no longer evident, the lobules are atrophic, and there are more small areas of regeneration. Subsequently, the lesion does not appear to progress further, but becomes modified by an increasing amount of active parenchymal regeneration.

From these bases, established by the killing and examination of the (b) groups, the effects of the second course of injections of alkaloid on the lesion already existing, at the time the injections were started, can be determined in the (a) groups at death. Broadly, the effects are to increase the degree of megalocytosis, the new bile-duct formation, and the cholangiole hyperplasia, and at the same time to cause the disappearance (or transformation) of small centrilobular hepatocytes and the appearance of hydropic degeneration, and to render atrophic changes more obvious.

In every instance the lesion is more chronic and further advanced in the (a) groups than in the corresponding (c) groups, although the mean total lethal dose was approximately the same. These results demonstrate very clearly that the morphological changes in the organ may not always be closely correlated with the lethal effect. In other words, the lethal result appears to depend more definitely on the biochemical than on the morphological nature of the lesion.

In groups 4(a) and 5(a) parenchymatous regeneration was well advanced at the time the second course of injections was started. Nevertheless, when the lethal effect was reached, after a mean period of only 64 and 56 days respectively, this regenerated parenchyma had almost completely disappeared; that

TABLE 2.

Nature and degree of heliotrine-produced liver lesion (i) at end of 1st course of injections, group 6(b); at time of second course of injections, (b) series; (iii) at death during second course, (a) series, and (iv) at death in control rats, given second course only, (c) series

		Liver lesions at time of slaughter									Liver lesions at time of death							
† Time (weeks)	Group	1 Meg.	2 NBDF	3 Chol. hyper.	4 Small cent. cells	5 Atrop. lob.	6 Reg.	7 Hydrop. deg.	8 Copper conc. p.p.m.	Group	9 Meg.	10 NBDF	11 Chol. hyper.	12 Small cent. cells	13 Atrop. lob.	14 Reg.	15 Hydrop. deg.	16 Copper conc. p.p.m.
0	6(b)	+	−	−	+ + +	−	−	−	29	..	..	..	..	..	..	..	..	..
10	1(b)	+ + +	+	−	+	−	−	−	76	1(a)	+ + + +	+ + +	+ +	±	+	−	+ +	243
..	..	..	..	..	..	..	..	..	..	1(c)	+ + +	+ +	+ +	+ +	±	−	−	272
15	2(b)	+ + +	+ +	+	+	−	±	−	35	2(a)	+ + + +	+ + +	+ +	−	+ +	−	+ +	308
..	..	..	..	..	..	..	..	..	..	2(c)	+ +	+	−	+ +	±	−	+	232
20	3(b)	+ + + +	+ +	+ +	−	+	+ +	−	28	3(a)	+ + + +	+ + +	+ +	± ‡	+ +	± •	+ +	310
..	..	..	..	..	..	..	..	..	..	3(c)	+ +	+	−	+ + +	±	−	±	240
25	4(b)	+ + + +	+ +	+ +	−	±	+ + +	−	24	(4a)	+ + + +	+ + +	+ +	+ ‡	+	± •	+	221
..	..	..	..	..	..	..	..	..	..	4(c)	+ +	+	−	+ + +	±	−	+	213
30	5(b)	+ + + + §	+ + §	+ §	−	−	+ + + +	−	19	5(a)	+ + +	+ +	+	+ ‡	±	± •	+	182
..	..	..	..	..	..	..	..	..	..	5(c)	+ +	+	−	+ + +	±	−	±	257

† Intervals between time of completion of first course of injections and the times at which the (a) groups were started on the second course and at which the (b) groups were killed.

• Remnant of regeneration.

‡ Possibility from earlier regeneration.

§ Confined to old damaged area: regeneration 30-50 p.c. of liver mass.

Abbreviations in columns should read—1 Megalocytosis, 2 New bile-duct formation, 3 Cholangiole hyperplasia, 4 Small centrilobular cells, 5 Atrophic lobules, 6 Regeneration, 7 Hydropic degeneration, 8 Copper concentration. These are repeated in the columns 9-16.

is, the new regenerated cells had undergone a megalocytic change and some may have died. It is characteristic of the (c) groups, which received an uninterrupted course of injections, that parenchymal regeneration did not occur. This is in complete agreement with all our previous experimental experience with rats on heliotrine at the dose rate used in this experiment.

Of the so-called toxic degenerations, fatty metamorphosis was almost completely absent in the livers. Hydropic degeneration in vesicular form was strongly evident in groups 1(a), 2(a) and 3(a), but much less evident in groups 4(a) and 5(a). It was absent from the livers of group 1(c), and was in slight evidence only in the other (c) groups. Although hydropic degeneration is generally characteristic of the terminal lesion, it is clear that the function of the organ may fail without this vesicular degeneration becoming manifest.

The significance of the high concentration of copper in the livers of the rats that died from the chronic liver disease is not understood, but it may be one expression of the altered metabolism of the hepatocytes and of the biochemical lesion.

A few of the rats which received the first course of injections died before they could be included in the particular group to which they had been allotted. These represented the most highly susceptible rats. Four of them died 59, 94, 111, and 196 days after the last injection in the first course. The pathological lesion was well advanced in all these rats, well beyond the lesions in the (b) groups killed at approximately the same time. Further, the liver-copper concentrations were 265, 940, 521 and 186 p.p.m., whereas the mean values in the (b) groups fell gradually during the same period of time from 75 to 19 p.p.m. These incidental observations add to the weight of experimental evidence on the progressive nature of the lesion produced by the action of the alkaloid.

DISCUSSION.

The results of the experiment show beyond reasonable doubt that heliotrine, as an example of one of the hepatotoxic pyrrolizidine alkaloids, when given to rats in small doses repeated over a period of time, will produce at the same mean total dose a lethal effect from chronic liver disease whether or not there is a break in the continuity of the dosage, and irrespective of the length of the break up to 30 weeks. They show also that there is a rise in susceptibility with increasing age of the rats.

This phenomenon, this function of total dose in relation to lethal effect as a summation of repeated small doses of heliotrine, was revealed on analysis of the data from the experiment. Druckrey (1959) has described a similar phenomenon in his study of carcinogenesis. He says that "Unlike all other pharmacological actions the carcinogenic action studied here does not depend on the individual daily doses but is determined by total dose". We are interested to know that this phenomenon is, according to Druckrey, unlike all other pharmacological actions. However, in our studies on the chronic liver disease

produced in larger domestic animals and in rats we have so far found no evidence of neoplastic changes.

It is important to remember in these respects that dose is measured in terms of the acute LD_{50}, strictly in relation to body weight.

It should also be noted that in these studies we are concerned with the chronic disease produced by repeated small doses. The methods used exclude necrotic and other effects which may be more strictly toxic in nature due to high dose levels. Thus, by these methods we obtain mainly the long-range effects of the action of the alkaloid on the nucleus of the hepatocyte, which may, of course, be secondary to specific enzyme changes in the cytoplasm.

The increased susceptibility of older rats to the chronic effects of the alkaloid appears to be in some conflict with the results obtained by Schoental (1959) who found with retrorsine and other pyrrolizidine alkaloids that susceptibility of rats decreased with age. She used the oral method of administration and larger doses about the LD_{50}, and thus was dealing with the more acute lethal effects. Further, in her control groups she had a death rate of approximately 50 p.c. over a period of 30 days. The very young rats were seriously disturbed by handling, but also they possibly absorbed the alkaloid through the alimentary canal more efficiently than older animals. However, we have not found that young rats 30 days old are more susceptible to acute toxic death from single intraperitoneal injections of heliotrine than older rats from 120 to 320 days old.

The mechanism of the production of a lethal lesion is not yet fully revealed. We have long been struck by the characteristic megalocytosis of the liver produced at least in sheep, horses and rats by the action of these alkaloids (Bull, 1955). We believe that this megalocytosis is an example of polyploidy in an organ in which the cells normally undergo some degree of polyploidy with advancing age. However, from visual appraisal of cytological changes in the hepatocytes, by the aid of the Feulgen technique, in the lesions produced the correlation between volume of nucleus and DNA content is not entirely regular, but there can be no doubt that the amount of DNA in the enlarged nuclei is usually increased significantly beyond that in the diploid nucleus. More exact quantitative studies have not yet been completed.

It is postulated that the megalocyte undergoes some changes in its metabolism, is possibly more susceptible to the alkaloid, and is more senile with a shorter life expectancy. Thus loss of tissue results, atrophic changes occur, and the bile-duct system becomes hyperplastic in an attempt to replace lost tissue.

The nature of the pathological changes suggests that these pyrrolizidine alkaloids act in a manner similar to some of the mitotic poisons. We have found with the aid of colchicine, that in the regenerating liver of the rat after partial hepatectomy, liver-cell division and multiplication are most active in the midzonal and peripheral zones of the lobule. This may explain partly or fully the early zonal distribution of the megalocytic response in the liver to these alkaloids. The hepatocytes in these zones may be more susceptible than those in

the centrilobular zone because they are nearer the stage of active division. So far the evidence does not suggest that active mitosis is necessary for the development of susceptibility to the action of the alkaloid.

It seems that something in the nature of a mutation occurs in the hepatocytes and that this is transmitted to the succeeding generations. Thus the nucleus increases in size long after the administration of a large single dose or of repeated small doses has ceased. Further, in groups 4(b) and 5(b) a great deal of regeneration of parenchyma had occurred 25 and 30 weeks after the last dose of alkaloid. Nevertheless, in groups 4(a) and 5(a) these new cells, the nuclei of which resembled diploid nuclei, relatively quickly became enlarged so that at death of the rats there was little or no recognizable recent regenerated tissue. Possibly they inherited an increased susceptibility to the alkaloid. This would be consistent with the finding that the mean total lethal dose is approximately the same irrespective of the degree of regeneration that has occurred.

Acknowledgments. We are indebted to Mr. W. R. Flower, Division of Mathematical Statistics, C.S.I.R.O., for the statistical examination of the data, and to Mr. M. W. Mules for assistance with injections and necropsies of the rats.

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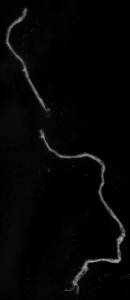
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